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COPROPORPHYRIN III ISOLATED FROM THE HUMAN TUBERCLE BACILLUS BY CHROMATOGRAPHIC AND FLUORESCENCE ANALYSIS. FLUORESCENCE AND ABSORPTION SPECTRAL DATA.

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PORPHYRINS are present both in hemoglobin and chlorophyll, and are of importance in the formation of many plant and animal pigments. The framework of the porphyrin molecule was established by Fischer, by Küster, and by Schumm, 1912–1935. There are four possible isomeric forms of the simplest porphyrin nucleus—etioporphyrin, designated by Fischer as etioporphyrin Types I, II, III and IV. Hence, he classified other porphyrins according to which types of etioporphyrin their structures corresponded. Porphyrins of Types I and III have been found in nature, and those of Types II and IV have been obtained only artificially.

Fischer (1916) isolated from the urine and faeces of the Petry case of congenital porphyrinuria porphyrins corresponding to etioporphyrin Type I, and named them uroporphyrin I and coproporphyrin I. Coproporphyrin III was also isolated by Fischer and Duesberg (1932) and Van den Bergh (1928) from other cases of the same disease. Grotepass (1932) isolated coproporphyrin III from the urine of a case of lead poisoning. Later, Grinstein, Schwartz and Watson (1945) and others investigated the occurrence of isomers Types I and III in cases of various diseases (review in *Annual Review of Biochemistry*, 1950, xix, p. 431).

Fischer (1930*a*) established the fact that organisms of both plants and animals are capable of synthesizing both isomeric types of porphyrin. This evidence led Fischer to enunciate his concept of the " dualism of the porphyrins " in nature (1930*b*). This principle has since been confirmed by numerous investigators. Fischer also determined that it is chemically inconceivable for one type to be transformed into the other without complete destruction and resynthesis.

Fischer and Fink (1925) reported the absorption spectrum of a metal complex of coproporphyrin extracted from heat-killed tubercle bacilli (maxima λ5226 and 5604Å). Dhéré and Rapetti (1935) reported the fluorescence spectrum of coproporphyrin in cultures of mycobacteria, including those of human tubercle bacilli grown on slants of solid media, with bands that varied in position between

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λ6295 and 6205Å in different samples. The type of coproporphyrin was not designated in any of these reports. Todd (1949) has recently reported the occurrence of coproporphyrin III in *Mycobacterium* sp. *karlinski*.

We present evidence in this report that coproporphyrin III has been isolated from virulent human tubercle bacillus strain No. 48189 grown in synthetic medium, which to our knowledge has not previously been reported for this species.

MATERIAL AND METHODS.

Strain No. 48189 was isolated from gastric contents collected July, 1947, and used in the preparation of antigens for complement-fixation tests. The tubercle bacilli were grown for eight weeks in Sauton's medium, which consists of: asparagine (Pfanstiehl), 4 g.; glycerol (Merck), 75 g.; citric acid, 2 g.; K_2HPO_4, 0·5 g.; $MgSO_4$, 0·5 g.; ammoniated iron citrate, 0·05 g.; distilled water, 925 g.

The source of the material for which fluorescence and absorption spectral data and other physical characteristics are presented was an alcoholic extract of heat-killed human tubercle bacilli (100 g. dry weight). Distilled water was added to the extract to make an alcoholic solution of 70 per cent, which was partitioned with a benzine-ether 1 : 1 mixture. The red fluorescing material in the red-tinted alcoholic solution passed into the benzine-ether phase, which then became red in daylight. Some samples of this solution were evaporated to dryness and the residues were dissolved in various solvents, such as acetone, ether, alcohol, chloroform, and hydrochloric acid, in order to determine the most suitable solutions for chromatographic separation and purification of constituents. Other samples of the benzine-ether phase were partitioned with 1·5 N HCl. The red fluorescing material now in hydrochloric acid solution was next transferred to ether. This was accomplished by neutralizing the solution to congo red with sodium acetate, and treating it with acetic acid to make a 10 per cent acetic acid solution. This transfer was usually repeated several times, and the red fluorescing ether solution was added to an equal volume of benzine because this mixture was found to be more suitable for chromatographic analysis than the ether solution alone.

Numerous experiments were performed in the chromatographic separation and purification of the constituents of these solutions on columns of aluminium oxide (Al_2O_3) standardized according to Brockmann and Schodder (1941) by Merck, hereafter called alumina. The chromatographs were usually short, 6 to 10 cm. high, and 0·7 cm. in diameter. Only when a small amount of pigment in benzine-ether 1: 1 solution was adsorbed on the alumina to a distance of about one-fifth of its length was the subsequent development of the chromatograph with suitable elutriants satisfactory in separating the zones as they passed down the column. The solutions elutriated from these zones were passed through fresh columns of alumina for further purification.

Observations of the chromatographs when subjected to the radiations from a Hanovia analytic mercury lamp have revealed zones, some not visible in daylight, that fluoresced with red, yellow, green, blue, violet and purple light. Usually four or five red fluorescing zones were noted. Since, obviously, the amount of material obtained from most of these zones was very small, attention was concentrated upon the solution designated as Fraction A obtained from the lowest red

fluorescing zone on the column that usually yielded sufficient material for determination of some of its characteristics.

Samples of the Sauton's medium were treated in a similar manner; but spectroscopic and fluorescence tests for porphyrin were negative.

Fluorescence and absorption spectral data are reported for some of the purified fractions. The fluorescence spectrograms were recorded on Kodak panchromatic and spectroscopic Type 1L plates, red sensitive, with a Hilger medium quartz spectrograph E 316. The slit width was 0·15 mm. Radiations from a Hanovia analytic mercury lamp were used to excite the fluorescence in samples contained in glass cells, which were placed approximately 1 cm. before the slit of the spectrograph and 10 cm. from the lamp. Helium was the reference spectrum. The spectrograms were evaluated by tracings made with a Zeiss recording microphotometer. Measurements of the tracings for wave-length determination were made with a Hilger measuring micrometer.

Spectrophotometric data in the ultraviolet region were recorded on Kodak spectroscopic plates Type 1L, Kodak panchromatic and Kodak 33 plates with the quartz spectrograph and Spekker photometer. The light source was a tungsten steel condensed spark. The slit width was 0·04 mm. Absorption data in the visible region were obtained with a Hilger-Nutting spectrophotometer.

RESULTS.

A sample of the benzine-ether 1 : 1 phase of the original alcoholic extract was placed on a column of alumina, and the material in the lowest red fluorescing zone, Fraction A, was then elutriated with benzine-ether. This zone was usually in close juxtaposition with a lower dark purple-brown fluorescing zone, which was orange-red in daylight, and had chromatographic characteristics similar to a zone of an erythropterin-like pigment described in an earlier report (1949). Hence it was necessary to further purify the material on a fresh column of alumina, and the red fluorescing material, Fraction B, thus was separated from the purple fluorescing material.

The microphotometric tracing of the fluorescence spectrogram of the purified red fluorescing benzine-ether solution, Fraction B, indicated a band with a maximum at λ6211Å, which agrees with Dhéré's value for coproporphyrin stated above.

A sample of the benzine-ether 1:1 phase was partitioned with 1·5 N HCl into which the red fluorescing material was transferred. The hydrochloric acid solution, Fraction C, was neutralized to congo red with sodium acetate, and acetic acid added to make the solution 10 per cent, and then treated with ether, which was washed with distilled water. This red fluorescing ether solution, Fraction D, was not passed through a chromatograph.

The microphotometric tracing of the fluorescence spectrogram of this ether solution, Fraction D, is presented in Fig. 1. Two bands are shown with maxima at λ6926 and 6200Å, which agree with those reported by Bois (1927) for coproporphyrin.

The benzine-ether solution that was passed through the second chromatograph to yield the material, Fraction B, for which the fluorescence maximum was reported above, was highly coloured and nearly matched Munsell* colour chip

* Information about the Munsell System of Color Notation may be found in the publications of the Munsell Color Company Inc., 10E. Franklin Street, Baltimore ,Maryland, U.S.A.

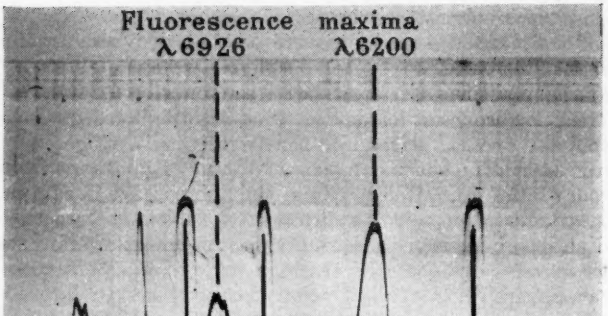

FIG. 1.—Microphotometric tracing of fluorescence spectrogram of coproporphyrin III isolated from human tubercle bacilli. Fraction D (ether solution).

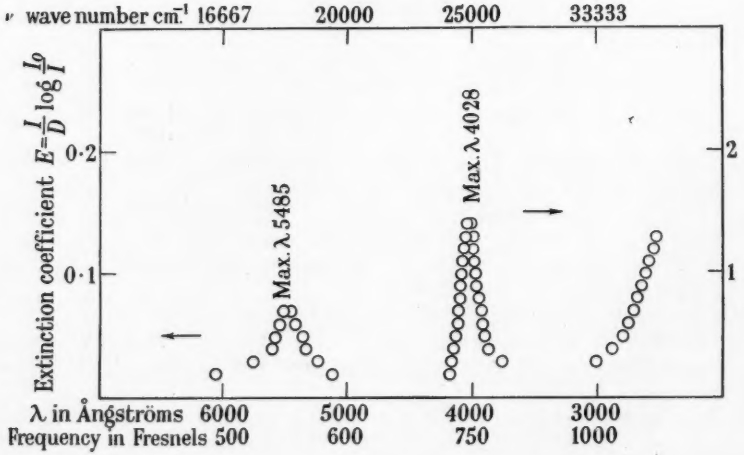

FIG. 2.—Ultraviolet absorption spectrum of coproporphyrin III isolated from human tubercle bacilli. Fraction C (1·5 N HCl).

RP-R 6/10 (red purple-red 6/10) by transmitted daylight through a depth of 1·5 cm. This solution produced in a depth of 1 to 2 cm. absorption bands with maxima at λ6246, <5746, <5323, <4958Å with increasing intensities as indicated. The ultraviolet absorption curve showed a band maximum at λ4053Å. When this solution was further purified by passing it through a third column of alumina to yield Fraction E, its absorption spectrum curve had a band maximum at λ4066Å in benzine-ether solution.

Before the transfer of its red fluorescing pigment into ether solution, Fraction D, which yielded the fluorescence recorded in Fig. 1, the 1·5 N HCl solution, Fraction C, produced absorption with band maxima at λ5485 and 4028Å, Fig. 2. These bands fall within the range of values reported by Jope and O'Brien (1945) for HCl solutions of coproporphyrin III in concentrations of 0·02 N to 7 N.

Crystallization was difficult to attain without recourse to methods of precipitation and saponification which require larger quantities of material than were available. However, a few ether solutions on standing for a few days yielded fine reddish prismatic needles, usually clustered in the form of rosettes. The melting point, obtained with a Kofler apparatus, was at 150·2° C. and, after cooling, the remelting point was at 170° to 171° C. These values fall within the range of melting points reported for coproporphyrin III from natural sources. A photomicrograph of the crystals (× 85) is presented in Fig. 3.

FIG. 3.—Photomicrograph of crystals (×85) of coproporphyrin III isolated from human tubercle bacilli.

Although the absorption spectra for coproporphyrins I and III are very similar, their crystal forms and melting points are sharply differentiated. The Type III isomer has the same rosette formation as recorded in Fig. 3 for our material and a double melting point.

SUMMARY.

Fluorescence and absorption spectral data have been presented for fractions of an alcoholic extract of human tubercle bacilli. The emission and absorption band maxima are within the ranges reported for coproporphyrin III. The crystal forms from these fractions and their melting points conform to those of coproporphyrin III. Hence the evidence presented indicates that coproporphyrin III is synthesized by the human tubercle bacillus.

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NUCLEIC ACID METABOLISM IN VIRUS INFECTIONS.

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THE virtual absence of enzymes from viruses suggests that the substances required for the formation of new viral material must be provided by the activity of the enzymes of the host cell, and a study of the biochemical activities of tissues during the course of a virus infection may therefore reveal the presence of abnormalities of metabolism which might afford some evidence of the way in which virus multiplication is brought about. In previous work it has been shown that tissues infected with viruses show an increased dehydrogenase activity as compared with normal tissues (Bauer, 1947*a*, 1947*b*; 1948). Mouse brain infected with the viruses of yellow fever, lymphocytic choriomeningitis and lymphogranuloma inguinale was found to possess an increased xanthine oxidase activity, while the oxidation of pyruvate was also increased, and an increase of xanthine oxidase was also found in chick embryos infected with yellow fever virus. Since nucleoproteins form an important part of the constitution of viruses, while the synthesis of nucleoprotein seems to be an essential part of virus multiplication, it was considered that a study of the activity of enzymes concerned in nucleic acid metabolism might be of interest, and might provide some evidence of the nature of the metabolic pathways which are traversed by the normal cellular constituents in the course of their incorporation into virus material. The viruses employed in the present study were chosen for their ability to multiply in the same tissue, mouse brain, and the object of the work was first to investigate the enzyme activity of normal mouse brain and establish a set of normal values, and subsequently to investigate the activity of the same enzymes in the same tissue during the course of various virus infections, and to note any deviations from the previously established normal values.

METHODS.

Viruses.

The viruses used were neurotropic yellow fever, lymphocytic choriomeningitis, a strain of vaccinia adapted to mouse brain (strain IHD, supplied by Dr. Randall Thompson, of the Indiana University Medical Centre, U.S.A.), and dengue (the Hawaii strain, supplied by Dr. A. B. Sabin, of the Children's Hospital Research Foundation, Cincinnati, U.S.A.). The viruses were passaged regularly to maintain constant titre and virulence; the first two viruses regularly attained a titre of 10^6 to 10^7, vaccinia a titre of 10^5, while the titre of dengue virus ranged from 10^4 to 10^5.

Enzyme Determinations.

General.

Brains were removed from normal mice, or from mice showing typical symptoms of disease, and were ground in a mortar with sufficient buffer solution to make a 10 per cent suspension. The composition and pH of the buffer varied in different experiments, and depended upon the nature of the enzyme reaction being investigated. The extract was clarified by low speed centrifugation, and the supernatant fluid was removed and used for the determination of enzyme activity. In the case of each enzyme a series of preliminary experiments was carried out to determine suitable concentrations of extract and substrate, and a suitable pH and period of incubation. Once established, these values were adopted as a routine for the actual determinations.

Three groups of enzymes were investigated, namely desaminases, nucleosidases and nucleotidases.

Desaminases.

Those investigated were guanase and the desaminases for adenosine and guanosine.

Guanase.—A brain extract was made with M/10 phosphate buffer of pH 8·0; 1 c.c. was added to 1 c.c. of a suspension of guanine containing 2 mg. per c.c. The mixture was incubated at 37° C. for 45 minutes.

Adenosine desaminase.—The extract was made with M/10 phosphate buffer of pH 7·0; 1 c.c. was added to 1 c.c. of a solution of the substrate containing 2 mg. per c.c., and the mixture was incubated at 37° C. for 120 minutes.

Guanosine desaminase.—This enzyme was determined in a similar manner to adenosine desaminase, except that incubation was carried out for 30 minutes.

In all the desaminase determinations blanks were set up, consisting of 1 c.c. of brain extract with 1 c.c. of distilled water.

After incubation 4 c.c of 3·3 per cent. trichloracetic acid were added to both test and control mixtures, and the precipitate of protein was filtered off. The concentration of the reaction product, ammonia, was determined in an aliquot of the filtrate by an adaptation of Conway's microdiffusion method.

Nucleosidases.

An extract of mouse brain was made in M/10 phosphate buffer of pH 7·0; 2·5 c.c. of the extract were added to 2·5 c.c. of a solution of nucleoside containing 2 mg. per c.c. Preliminary experiments showed that a long period of incubation was necessary, and 4 drops of chloroform were therefore added to the mixture to prevent the growth of bacteria. Incubation was carried out at 37° C. for 5 hours in experiments with guanosine, and for 24 hours with inosine, uridine, guanosine, xanthosine and adenosine. A few experiments were carried out with cytidine, which was found to be unattacked. After incubation, proteins were precipitated by the addition of 2·5 c.c. of 2/3 N sulphuric acid, and 2·5 c.c. of 10 per cent sodium tungstate, and were removed by filtration. The ribose liberated during the course of the reaction was determined in an aliquot of the filtrate by Somogyi's modification of the Schaffer-Hartmann method (Somogyi, 1926). Blank determinations were found to be unnecessary, as the brain extracts

contained little or no reducing material, and no decomposition of the substrate occurred during the period of the reaction.

Nucleotidases.

Brains were extracted with pH 9·0 veronal buffer containing magnesium ions in a concentration of 2×10^{-3} M; 1 c.c. of the extract was added to 1 c.c. of a solution of guanylic acid or adenosine-5-phosphoric acid containing 2 mg. per c.c. A blank was prepared in each case, consisting of 1 c.c. of brain extract and 1 c.c. of distilled water. The mixtures were incubated at 37° C. for 60 minutes.

Adenosinetriphosphatase.—Brains were extracted with pH 7·0 veronal buffer, and the extract was further diluted with the same buffer to make a 2 per cent extract; the use of a higher dilution was made necessary by the great activity of the enzyme. One c.c. of the diluted extract was added to 1 c.c. of a solution of the calcium salt of adenosinetriphosphoric acid containing 2 mg. per c.c., and the mixture was incubated at 37° C. for 30 minutes. Similar tubes containing an excess of potassium oxalate to precipitate calcium ions were set up as blanks. As adenosinetriphosphatase is activated by calcium ions the subtraction of the blank values gives the amount of phosphate liberated by the enzyme. The reaction was carried out at pH 7·0 to reduce to a minimum the amount of phosphate liberated by the alkaline phosphatase also present in the extract.

At the end of the reaction period in the nucleotidase and adenosinetriphosphatase determinations 4 c.c. of 3·3 per cent trichloracetic acid were added to precipitate proteins. After filtration the phosphorus contact of an aliquot of the filtrate was determined by the method of Fiske and Subbarow (1925); subtraction of the blank values gave the amount of phosphorus liberated by the action of the enzymes under investigation.

RESULTS AND DISCUSSION.

The results are shown in Table I. It will be seen that, whereas enzyme activity is often unchanged, it is in certain instances increased or decreased in the presence of a virus infection. It is, of course, not remarkable that an enzyme activity should be reduced in the brain of an animal moribund from encephalitis, nor even that an enzyme activity should show no difference from normal values, but the finding that an enzyme activity can actually be increased in a virus infection represents a point of interest for further investigation, and a possible guide to an understanding of the processes by which virus multiplication is brought about. As has been pointed out previously (Bauer, 1947*a*, 1947*b*), a round cell infiltration occurs in virus encephalitis and, before conclusions can be drawn, it must be clearly shown that the observed increase of enzyme activity is not brought about by the immigration of leucocytes. It has already been shown that the increase of dehydrogenase activities in virus encephalitis is not due to the enzyme activity of leucocytes, since the activity of other enzymes which can be shown to be present in leucocytes remains unaffected. A similar method was employed in the present study. Extracts of lymph glands, made by the same method as was used for mouse brain, possessed high guanase and guanosine desaminase activity. As shown in Table I, guanase activity is unaffected in virus infection, while the activity of guanosine desaminase may be unaffected or decreased. It is therefore evident that the activity of these two enzymes

TABLE I.—*Enzyme Activities in Mouse-brain Extracts Expressed as Percentage of Completion of Reaction in One Hour.*

Enzyme.	Virus.	Number of determinations.	Mean of determinations.	Standard deviation.	Difference from normal.	P.	Extent of change expressed as fraction of normal value.
Desaminases :							
Guanosine	Control	17	69·92	9·922	..	..	..
	NYF	12	64·51	5·504	Unchanged	..	..
	LCM	12	57·75	12·42	„	..	..
	NV	12	55·49	9·036	Decreased	0·05–0·02	−0·11
	D	12	55·46	10·21	„	0·05–0·02	−0·11
Adenosine	Control	11	11·47	3·760	..	..	..
	NYF	13	14·49	0·5399	Increased	0·075	+0·27
	LCM	12	15·27	3·542	„	0·02	+0·33
	NV	12	15·63	4·063	„	0·01–0·001	+0·37
	D	13	24·90	6·868	„	<0·001	+0·17
Guanase	Control	10	23·55	3·784	..	..	..
	NYF	8	21·52	3·474	Unchanged	..	..
	LCM	10	24·00	3·639	„	..	..
	NV	10	23·16	4·078	„	..	..
	D	10	22·29	5·027	„	..	..
Nucleosidases :							
Inosine	Control	20	2·292	0·3958	..	..	..
	NYF	22	2·705	0·3888	Increased	0·01–0·001	+0·18
	LCM	14	2·667	0·2659	„	0·01–0·001	+0·16
	NV	17	2·072	0·3747	Unchanged	..	..
	D	12	2·865	0·3994	Increased	<0·001	+0·27
Uridine	Control	12	1·031	0·1296	..	..	..
	NYF	12	1·451	0·4547	Increased	<0·001	+0·40
	LCM	12	1·300	0·1269	„	<0·001	+0·26
	NV	12	1·345	0·2494	„	0·01–0·001	+0·21
	D	12	1·972	0·2598	„	<0·001	+0·91
Guanosine	Control	17	9·679	2·012	..	..	..
	NYF	16	9·679	1·796	Unchanged	..	..
	LCM	12	9·296	1·689	„	..	..
	NV	12	9·545	1·689	„	..	..
	D	12	13·680	1·121	Increased	<0·001	+0·89
Xanthosine	Control	16	1·283	0·385	..	..	..
	NYF	14	1·620	0·4811	Increased	0·05	+0·26
	LCM	12	1·579	0·4112	Unchanged	..	..
	NV	19	1·431	0·1279	„	..	..
	D	11	1·752	0·2167	Increased	<0·001	+0·36
Adenosine	Control	11	2·220	0·2503	..	..	..
	NYF	12	2·325	0·3237	Unchanged	..	..
	LCM	16	2·205	0·8424	„	..	..
	NV	22	2·313	0·07577	„	..	..
	D	12	2·743	0·1362	Increased	<0·001	+0·24
Nucleotidases :							
Adenosine-5-phosphoric acid	Control	12	8·985	3·089	..	..	..
	NYF	13	7·528	1·408	Decreased	<0·001	−0·17
	LCM	17	8·930	1·669	Unchanged	..	..
	NV	10	10·350	0·6725	Increased	0·05–0·02	+0·18
	D	13	9·141	3·620	Unchanged	..	..
Guanylic acid	Control	15	5·978	0·6607	..	..	..
	NYF	19	4·435	1·137	Decreased	0·05–0·02	−0·36
	LCM	15	6·915	1·341	Unchanged	..	..
	NV	17	7·142	2·224	„	..	..
	D	11	5·939	1·104	„	..	..
Adenosinetriphosphatase	Control	16	36·14	10·440	..	..	..
	NYF	12	38·14	9·363	Unchanged	..	..
	LCM	11	32·38	8·030	„	..	..
	NV	16	60·58	18·036	Increased	<0·001	+0·68
	D	16	30·52	21·66	Unchanged	..	..

NYF = Neurotropic yellow fever ; LCM = lymphocytic choriomeningitis ; NV = neurovaccinia ; D = dengue.

contributed by the round-cell infiltration is too small to be detected, and it necessarily follows that the round-cell infiltration is not responsible for the increase in other enzyme activities. There is a second possibility—that the increased enzyme activity is due to an enzyme property of the virus particles themselves. The evidence for the possession of enzymes by viruses has already been reviewed in detail (Bauer, 1949), and it is concluded that there is no real evidence that viruses have any enzyme activity at all. The increase of enzyme activity found in mouse brain during a virus infection also bears no likely relation to the mass of virus material actually present. Thus, a brain infected with neurotropic yellow fever virus to a maximum titre of 10^7 contains only $0{\cdot}01\gamma$ of active virus at a rough estimate, yet in this infection the activity of uridine nucleosidase, for example, is increased by 40 per cent, a figure which is equivalent to the activity of $16{,}000\gamma$ of normal mouse brain. If this increase were due to an enzyme property of the virus itself, the virus would necessarily have an enzyme activity 1,600,000 times greater than that of a representative normal tissue, which seems highly improbable.

The third possibility, that the presence of the multiplying virus causes an increase in activity by exerting an influence upon the metabolism of the host, is of much greater interest, and this possibility becomes a strong probability by the dismissal of the other two. The increase of enzyme activity is probably a unique occurrence in infective conditions, and gives support to the view that viruses are unrelated to bacteria, and are agents which can produce effects which belong properly to the province of normal cytoplasmic constituents. The virus must exert an overriding control over the host enzymes which overcomes their natural restraints. There probably exists a balance between inhibition and activation of the host enzymes, which can be deflected in either direction according to metabolic needs, and which is selectively upset by the action of the virus. Such an action has already been postulated (Bauer, 1949), and it is suggested that the virus particles, after entering the host cell, dissociate into a soluble phase, which then diffuses through the cytoplasm and organizes the host metabolism in the directions required for the synthesis of virus material. There are several ways in which this might occur. There is some evidence that extra enzyme may be synthesized (Bauer, 1948), and viruses contain nucleic acids and polysaccharides, which might act as template molecules for the deposition of new protein. It is also possible that virus material might cause an increase in the activity of the necessary enzymes by the selective removal of inhibiting ions.

As has already been mentioned, a consideration of the behaviour of enzymes in the virus infections studied may give some indication of the metabolic processes involved in virus multiplication. The experimental results are shown schematically in Fig. 1 to 4, which represent the course of nucleic acid metabolism in the separate virus infections so far as the stage of production of nucleotides. Pyrimidine metabolism is the same in all cases; cytidine is not metabolized, while the activity of the nucleosidase for uridine is increased. Some variation occurs in the course of purine metabolism. In most cases an increased activity occurs in the pathways which lead from hypoxanthine through various intermediates to adenylic and guanylic acids. The results suggest that some of the existing metabolic pathways are quantitatively insufficient, and are selectively expanded under the influence of the virus.

A further fact which emerges from the results of Table I is that the pattern

Neurotropic Yellow Fever.

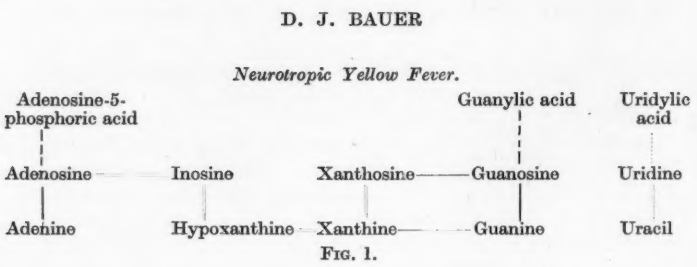

FIG. 1.

Lymphocytic Choriomeningitis.

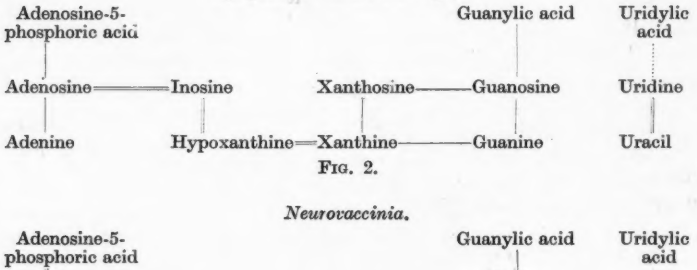

FIG. 2.

Neurovaccinia.

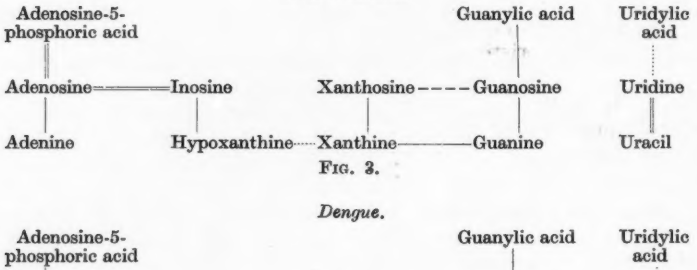

FIG. 3.

Dengue.

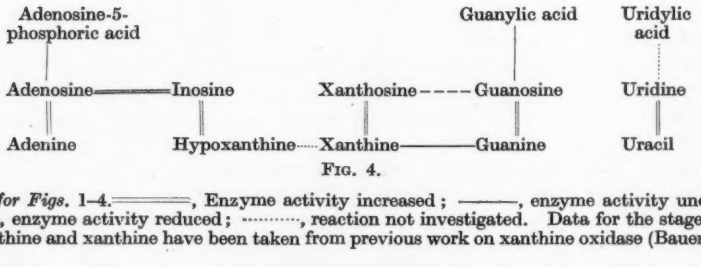

FIG. 4.

Notes for Figs. 1–4. ═══, Enzyme activity increased; ———, enzyme activity unchanged; – – – – –, enzyme activity reduced; ·····, reaction not investigated. Data for the stage between hypoxanthine and xanthine have been taken from previous work on xanthine oxidase (Bauer, 1947*a*).

of enzyme activation or inhibition is not the same in all the viruses. While there is a general agreement in the effects produced by yellow fever and lymphocytic choriomeningitis viruses, there is a considerable difference in the results obtained with vaccinia and dengue. A striking feature of vaccinia encephalitis, for instance, is the great increase in adenosinetriphosphatase activity, which is not found with the other viruses. Further study with other enzymes and viruses might perhaps reveal biochemical affinities between viruses which are not necessarily related serologically, analogous to the interference phenomenon which is often independent of serological affinities, and which might depend upon the mutual possession of a specific enzyme-organizing ability. Dengue seems to form a

special case, in that the changes in enzyme activity in this infection are in several cases of much greater extent than those produced by the other viruses. This is particularly noteworthy, since the titre of dengue virus is low, from 10^4 to 10^5, and it is surprising to find increases of enzyme activity occurring which are much greater than those found in neurotropic yellow fever and lymphocytic choriomeningitis, where there is 100 times as much virus present. The discrepancy could be explained if there happens to be much more virus present than can be detected by infectivity titrations; symptoms might appear when the cells are occupied by a fresh developmental cycle of virus which has not yet reached the stage of infectivity, but which is sufficient in amount to produce the rise of enzyme activity seen. A further point to be considered is that dengue virus is not well adapted to passage in mouse brain, and that the anomalous enzyme effect may be related to this in some way.

SUMMARY.

The activity of various enzymes concerned in nucleic acid metabolism has been investigated in normal mouse brain, and in the brains of mice infected with the viruses of yellow fever, lymphocytic choriomeningitis, vaccinia and dengue.

The activity of certain enzymes is increased in the presence of the virus infection.

The effect upon enzyme activity differs from one virus to another, and may perhaps be a characteristic of the virus concerned, and of other viruses related to it.

The identity of the enzymes which are affected gives some information about the metabolic processes which are concerned in the synthesis of new virus material.

The bearing of the results upon recent theories of virus multiplication is discussed.

Thanks are due to Dr. J. W. Trevan for the statistical evaluation of the results, and to Dr. R. J. C. Harris, of the Chester Beatty Research Institute, for the gift of a sample of uridine.

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AN EXPERIMENTAL STUDY OF SENECIO POISONING IN RATS.

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In another paper the findings in twelve cases of senecio poisoning in man are described (Selzer and Parker, 1951). Autopsies performed on the six fatal cases revealed ascites and a striking centrilobular haemorrhagic lesion in the liver which was shown to be due to occlusion of the hepatic veins (Chiari's syndrome). As the experimental production of Chiari's syndrome had not been reported by previous workers on senecio poisoning (Cushny, 1910 ; Theiler, 1919 ; de Kock, du Toit and Steyn, 1931 ; Davidson, 1935 ; Harris, Anderson and Chen, 1942), we decided to study afresh the lesions produced in rats by the senecio plant and its alkaloids, with special reference to any possible changes in the blood vessels of the liver. As our human cases were all persons in poor circumstances, some of our animals were placed on a low protein diet which was known to be effective in the production of massive hepatic necrosis.

METHODS.

In all experiments albino rats weighing from 60 to 120 g. were used.

Two diets were employed. The first was a normal stock cubed diet consisting of yellow maize meal 48 per cent, peanut meal 10 per cent, meat meal 15 per cent, fish meal 2·5 per cent, oatmeal 15 per cent, barley 7·5 per cent, lucerne 1 per cent, salts 0·5 per cent, and lime stone powder 0·5 per cent, which was supplemented by green vegetables twice a week. The second diet was a low-protein diet suggested by Gillman (1948, personal communication), and consisted of 90 per cent potato starch and 10 per cent food yeast, being prepared as follows : To 500 ml. of boiling water were added 90 g. of potato starch previously made into a smooth paste with 100 ml. of water. The mixture was stirred thoroughly and allowed to boil for 2 minutes. When cool, 10 g. of food yeast in 20 ml. of water were added and mixed in very thoroughly. This diet was administered to rats *ad libitum*, and was not supplemented by vitamins, green vegetables or salt mixtures.

The most suitable available senecio alkaloid was retrorsine, which is present in *Senecio ilicifolius*, although not the most plentiful alkaloid in that plant. It is present also in other senecio species. The alkaloid was supplied by Dr.

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H. L. de Waal, of Pretoria. Solutions were prepared by dissolving a weighed quantity of the alkaloid in an equimolecular amount of hydrochloric acid. This was administered by stomach tube, except in one experiment in which it was injected subcutaneously. In another experiment alcoholic and aqueous extracts of the plant, *Senecio ilicifolius*, were given by mouth.

The animals which did not die were killed by carbon monoxide gas in Experiment 1, and by dislocation of the neck in Experiments 2 to 6. Tissues were fixed in 10 per cent formalin, and paraffin sections were stained with haematoxylin and eosin and with van Gieson's stain. Frozen sections stained with scarlet red were examined for fat.

RESULTS.

Experiment 1 : Retrorsine hydrochloride 1 mg. per 10 g. body weight was administered in a single dose by mouth to 30 rats on a normal diet, and to 30 rats on a low protein diet. Of the 30 rats on a normal diet, 4 were dead by the third day, 8 died subsequently, and the survivors were killed on the twelfth day. In all the rats that died and in two of those killed on the twelfth day there was a haemorrhagic zonal necrosis of the liver. In the remainder the liver appeared normal.

Of the 30 rats which had been on a low protein diet for 21 days prior to the administration of retrorsine, 25 were dead 3 days after receiving the alkaloid, and 5 died within the next four days. Typical haemorrhagic zonal necrosis was found in 27 of the 30 rats. The low protein diet therefore appeared to enhance markedly the effect of the alkaloid.

Twenty control rats fed on a normal diet and housed under identical conditions did not develop any hepatic lesions. A second control group of 21 rats were fed a diet of food yeast and potato starch and did not receive retrorsine. Three died before the 35th day of the experiment, and these did not show any liver lesions. The remaining 18 rats died between the 35th and 70th days, and all showed a necrosis which was massive and not zonal in type.

At autopsy it was found that retrorsine produced similar hepatic lesions, both in the rats on a normal diet and in those on a low protein diet. Macroscopically the liver was tense and firm, with a uniform mottled appearance resembling that of passive congestion. Nine rats showed haemorrhage into the lungs, varying in size from minute petechiae to lesions involving more than half a lobe. Four of the animals had frank haemorrhage into the peritoneal cavity, the source of which could not be ascertained. In none was there marked oedema of the large bowel such as was seen in the human cases.

Histologically the liver damage was often more marked than the macrosopic appearance suggested. In all cases the lesion was essentially zonal, being most marked around the central vein, but frequently involving two-thirds of the liver lobule (Fig. 1). In the affected zones most of the liver cells had disappeared, and those that remained were necrotic. The same areas were filled with red blood corpuscles, so that the appearance suggested a recent haemorrhage. The picture contrasted markedly with that seen in the control rats fed a diet of food yeast and potato starch. The latter group of animals showed large areas of massive hepatic necrosis scattered throughout all the lobes, and separated by liver tissue which was normal or showed fatty change only. Histologically the appearance of massive necrosis was confirmed. Much of the necrotic tissue

was still *in situ*, although in some regions the dead cells had disappeared and the sinusoids were dilated.

Experiment 2: In an attempt to trace the evolution of the lesion produced by the senecio alkaloid, 43 rats on a normal diet were given retrorsine hydrochloride 1·5 mg. per 10 g. body weight in a single dose by mouth. Animals were killed at each three-hourly interval up to the 30th hour, and thereafter at frequent but irregular intervals up to the 72nd hour. Considerable variations were observed in the time of onset of the earliest demonstrable lesion and in the extent of the fully developed picture. Thus, whilst in some rats killed after 6 hours early lesions were seen, others killed 15 hours later showed a normal liver. Detailed results are recorded in Table I.

TABLE I.—*Experiment* 2.

Time in hours.	Rat.	Histological findings.
3	1 and 2	No abnormality seen.
6	3 and 4	No abnormality seen.
	5	Periportal sinusoidal dilatation.
	6	Centrilobular sinusoidal dilatation. Early degeneration of liver cells.
9	7	No abnormality seen.
	8	No abnormality seen except eosinophilic infiltration of portal tracts.
12	9, 10 and 11	No abnormality seen.
	12	Centrilobular haemorrhagic necrosis.
	13	Centrilobular haemorrhagic necrosis.
15	14	Slight centrilobular haemorrhagic necrosis.
18	15 and 16	Centrilobular sinusoidal dilatation. Infiltration with polymorphs.
21	17 and 18	No abnormality seen.
	19 and 20	Centrilobular haemorrhagic necrosis with disappearance of many liver cells. Infiltration with polymorphs.
24	21	Very extensive centrilobular haemorrhagic necrosis.
	22	Very slight centrilobular necrosis.
27	23 and 24	Occasional necrotic cells in central zones only.
30	25 and 26	Extensive centrilobular haemorrhagic necrosis. Heavy infiltration with polymorphs.
	27 and 28	No abnormality seen.
36	29 and 30	Extensive centrilobular haemorrhagic necrosis. Heavy infiltration with polymorphs.
	31 and 32	Extensive centrilobular haemorrhagic necrosis. Liver cells largely disappeared in central zones, which contain many macrophages. Slight "endothelial swelling."
48	33 and 34	Centrilobular "cysts." Slight "endothelial swelling."
52	35 and 36	Centrilobular "cysts" containing a few macrophages and polymorphs.
55	37	Centrilobular "cysts" containing macrophages and polymorphs. Slight "endothelial swelling."
60	38 and 39	Centrilobular "cysts" containing macrophages and polymorphs. Slight "endothelial swelling."
72	40 and 41	Centrilobular "cysts" containing macrophages. Slight "endothelial swelling."
	42 and 43	Slight centrilobular necrosis and "endothelial swelling."

The earliest change was seen at 6 hours. In two of four rats killed at this stage the liver appeared normal. The others showed dilatation of sinusoids, which was centrilobular in one rat and periportal in the other. In the former there was evidence of early degeneration of liver cells.

Two of five rats killed 12 hours after administration of the alkaloid showed a definite centrilobular necrosis and haemorrhage, so that in places there appeared to be pools of blood. The liver cells, though necrotic, were still *in situ*, and it was thus felt that the appearance was in fact a haemorrhage, and not a dilatation of sinusoids following on the removal of dead liver cells.

At 15 hours infiltration of the necrotic zone by polymorphs was first seen. This feature was noted in 9 out of 20 rats killed between 15 and 36 hours. At 21 hours disappearance of necrotic liver cells was obvious.

At 36 hours a vascular lesion similar to that described by Davidson (1935) in rats was first observed. It occurred in 10 out of 15 rats killed between 36 and 72 hours. The central and hepatic veins were often lined by a layer of large mononuclear cells, which Davidson considered to be swollen endothelial lining. In our animals these cells showed evidence of phagocytosis, and we therefore considered that they could perhaps be macrophages. This lesion was seen also in other experiments, occurring from 1½ to 12 days after administration of retrorsine. A further experiment with trypan blue was performed to elucidate this point and is described later.

From 40 to 72 hours after administration of the drug all the necrotic tissue in the central zones was absorbed, leaving sharply defined cyst-like spaces around the central veins (Fig. 2). These are referred to in Table I as " cysts."

Experiment 3 : Four rats were given 1 ml. of 1 per cent trypan blue subcutaneously on the first and the fourth days of the experiment, and retrorsine hydrochloride 1 mg. per 10 g. body weight orally on the fourth day. One rat died on the seventh day of the experiment, and the remainder were killed on the tenth day.

All but one of the rats showed the typical haemorrhagic zonal necrosis seen in the previous experiments, and, in addition, a marked lesion of the central and hepatic veins (Fig. 3–5). This consisted of one, two and sometimes even more layers of mononuclear cells, many of which contained trypan blue and showed other evidence of phagocytosis. Some of the cells were flattened against the wall, suggesting a mere swelling of the endothelial lining; others were attached by a broad base, and still others by an elongated cytoplasmic stalk (Fig. 5). Many of these cells were lying free in the lumen and similar cells were seen in the surrounding tissue.

The rat which did not show zonal necrosis had no evidence of a vascular lesion.

Experiment 4 : Several experiments were performed in an attempt, by varying the dosage and the route of administration of the alkaloid, to produce a vascular lesion approximating to that seen in the human cases.

Thus, 5 rats were given by mouth 0·05 mg. retrorsine hydrochloride per 10 g. body weight daily for 31 days. Five further rats were given 0·1 mg. daily for 21 days. Another group of 5 rats was given 0·5 mg. orally on alternate days for 7 days, by which time all 5 rats had died. In only one of these experiments was a lesion demonstrated in the hepatic veins.

Nine rats were then given retrorsine hydrochloride 0·5 mg. per 10 g. body

weight orally on the 1st, 2nd, 3rd, 10th, 11th, 12th, 30th and 31st days of the experiment and killed on the 35th day. Of these rats 5 showed early fibrosis commencing around the central veins, but in none was a vascular lesion demonstrable. It was felt that a more prolonged and perhaps more frequent administration of the drug in this dosage might have produced a more advanced picture of hepatic fibrosis.

Experiment 5: Ten rats were each given one subcutaneous injection of retrorsine hydrochloride 1 mg. per 10 g. body weight. One rat was killed 6 days later and showed a zonal haemorrhagic necrosis. The remainder were killed after 21 days. Two showed ascites, and in one of these there was also a pleural effusion. In those with ascites there was regeneration of liver tissue and early fibrosis, but no lesion was found in the central or hepatic veins.

Experiment 6: *Senecio ilicifolius* was gathered from the Knysna district, prepared in various ways and fed to rats. The experimental work was limited by the amount of plant available. Eight rats were given a normal diet to which the dried plant had been added, so as to form 10 per cent of the daily ration; 10 rats were given daily an aqueous extract of the dried whole plant in varying dosages; 6 rats were given daily an alcoholic extract prepared from the dried seed-heads of the plant. In these experiments the animals lived from 21 to 84 days. The results of these procedures were very variable and may be summarized as follows: Only 4 rats showed centrilobular haemorrhagic necrosis of the liver. In one of these and in three rats in which there was no necrosis there was a mild degree of apparent endothelial proliferation in the hepatic veins such as that which has already been described. In 5 rats a similar lesion was present in the pulmonary veins, and haemorrhages into the lungs were much more marked than in the previous experiments. These pulmonary haemorrhages were present in all but 3 animals. Ascites was present in 9 rats, 3 of which showed a zonal liver necrosis.

DISCUSSION.

Watt and Breyer-Brandwijk (1932), in discussing the pathogenesis of senecio poisoning, concluded that it was not possible to state whether the primary lesion was in the vascular system or in the liver parenchyma. More recent work has

EXPLANATION OF PLATE.

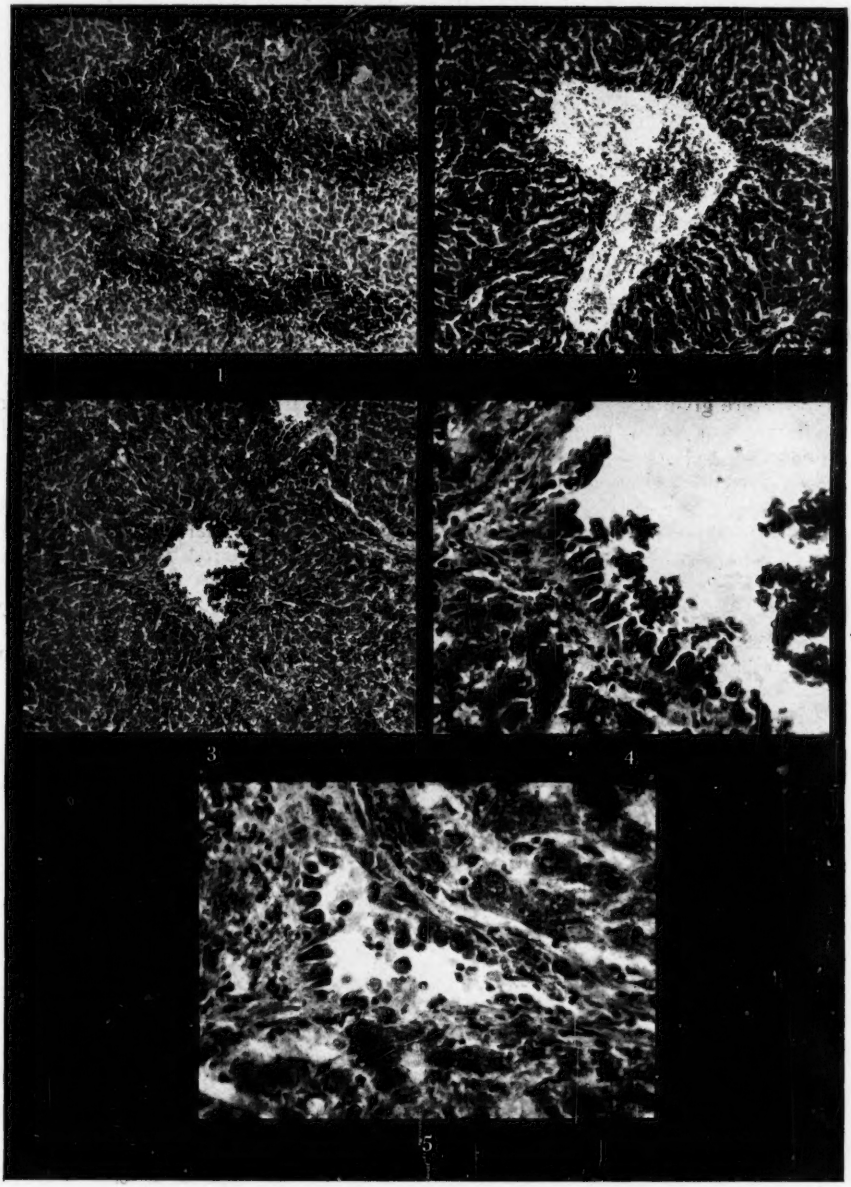
FIG. 1.—Liver of rat in Experiment 1 dying 2 days after administration of retrorsine. There is a zone of necrosis and haemorrhage around the central veins. Haematoxylin and eosin. × 100.

FIG. 2.—Liver from rat 40 in Experiment 2. The cells around the central veins have disappeared, leaving sharply defined cyst-like spaces. Haematoxylin and eosin. × 100.

FIG. 3.—Liver from rat in Experiment 3, killed six days after administration of retrorsine. A small hepatic vein is lined by a layer of mononuclear cells. Haematoxylin and eosin. × 100.

FIG. 4.—High-power view of the hepatic vein shown in Fig. 3. While some cells are lying free, others appear to be attached to the vessel wall by a cytoplasmic stalk. Haematoxylin and eosin. × 440.

FIG. 5.—Sublobular vein from the same animal as Fig. 2. The apparent attachment of the cells to the vessel wall is well seen, particularly to the left. Haematoxylin and eosin. × 440.



Selzer, Parker and Sapeika.

not changed the position appreciably. Davidson (1935), for instance, while describing both vascular lesions and hepatic necrosis, considers the former to be the primary. Rosenfeld and Beath (1945) come to the same conclusion. Harris, Anderson and Chen (1942), on the other hand, stress the liver necrosis, although they describe sinusoidal congestion and haemorrhage into cell cords.

The almost simultaneous appearance of parenchymal necrosis and haemorrhage in our second experiment suggests that, contrary to Davidson's view, there is a primary toxic effect both on the liver cells and on the vascular system. Indirect evidence in favour of this latter is the presence of haemorrhages in situations other than the liver observed by us and by other workers (Cushny, 1910 ; Rosenfeld and Beath, 1945). The early haemorrhages in the liver appear to result from rupture of the central and hepatic veins, but such disruption of the vessel walls as is seen could, it is felt, have been produced artificially in the preparation of the sections. The only constant and convincing vascular lesion is the apparent endothelial proliferation described in our second experiment. The lesion corresponds very closely to that noted by Davidson. He considered it to be a proliferation of the endothelial lining of central and hepatic veins. Alternatively, the cells lining the affected vessels might, especially in view of their phagocytic power as shown in Experiment 3, be macrophages swept in from the surrounding tissues. Their appearance, however, strongly suggests that they are produced locally by the vascular endothelium, and according to Gillman (1950, personal communication) the endothelium of the central and hepatic veins can be phagocytic. Whatever the origin of these cells, they would appear to be capable of blocking the vessels, and it is possible that, although we have not demonstrated such a further development of the lesion in our experiments, they may eventually be replaced by fibrous tissue. In this way the appearance seen in the human cases, and probably in horses (Theiler, 1919 ; de Kock, du Toit and Steyn, 1931), could be produced. At this late stage any primary damage to the liver parenchyma would be masked by the secondary effects of the vascular lesions. Whether such primary parenchymal damage occurs in man and horses it is not possible to say, for the earliest stages of the process in these species have not been described.

Finally, we have shown that a protein-deficient diet markedly enhances the effect of retrorsine. This may have a bearing on the aetiology of the human disease, as all the cases described in our previous paper came from families in poor circumstances.

SUMMARY.

1. The hepatic lesions produced in rats by retrorsine, a senecio alkaloid, are described.

2. The primary effect appears to be both on the liver parenchyma and on the central and hepatic veins, so that the picture of a centrilobular haemorrhagic necrosis is produced.

3. At a slightly later stage an apparent proliferation of the endothelium of the central and hepatic veins is seen.

4. The possible relation of the experimental to the human lesions is discussed.

We wish to thank Dr. C. Reineits, of Knysna, for gathering the senecio plant ; Dr. H. L. de Waal, of Pretoria, for the retrorsine ; Mr. D. Storey for preparing

the histological material ; and Professor G. C. Linder and Professor J. G. Thomson for their interest in this work.

We are especially indebted to Professor M. van den Ende for his helpful advice and criticism.

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OVOMUCIN: A SUBSTRATE FOR THE ENZYME OF INFLUENZA VIRUS. II. DEMONSTRATION OF ITS INHOMOGENEITY.

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IT was found previously (Gottschalk and Lind, 1949*a*) that of the various components of egg-white, only ovomucin is able to inhibit influenza virus haemagglutination, a property lost upon incubation with active viruses. In the same paper evidence was presented that ovomucin, as usually prepared, contains invariably lysozyme (up to 15 per cent). A further step in the differentiation of the ovomucin fraction is reported in the present communication. It was found possible to remove the inhibitory component from ovomucin almost completely, leaving behind the bulk of the mucoprotein. Erythrocytes, pre-treated with periodate and coated with influenza virus, provided a specific adsorbent for the haemagglutinin inhibitor, i.e., the substrate for the viral enzyme. It has been shown previously (Fazekas de St.Groth, 1949) that whereas red cells treated with higher concentrations of periodate do not adsorb virus at all, cells treated with between 2·0 and 0·2 mg. per g. of packed red cells bind virus firmly, so that it can neither be eluted nor liberated by the " receptor-destroying enzyme " of *V. cholerae*.

MATERIALS.

Ovomucin.

Ovomucin was prepared as described by Gottschalk and Lind (1949*a*) for the use in kinetic studies; for the adsorption experiments the ovomucin dispersion was diluted with saline to give a final concentration of about 0·2 per cent ovomucin and 1·2 per cent NaCl.

Viruses.

The classical influenza virus strains MEL (A) and LEE (B) were grown in the allantoic cavity by the method of Burnet (see Beveridge and Burnet, 1946). High titre allantoic fluids, free from bacterial contamination, were used either in the active state or after destruction of their enzymic activity by heating at 56° C. for 30 min. (pH 8·5) in the absence of Ca-ions (Stone, 1949).

Virus-coated red cells.

Human and fowl red blood cells were collected in physiological NaCl solution containing 1 per cent sodium citrate, and washed thrice in ten volumes of 0·85 per cent. NaCl solution.

Virus-coated cells were prepared as follows: packed red cells, pre-treated with an equal volume of M/1200 KIO_4, were mixed with ten volumes of high titre

virus preparation and allowed to settle at room temperature for 15 minutes. Then the supernatant was removed and the cells suspended in an equal volume of a preparation of the "receptor destroying enzyme" (RDE), obtained from *V. cholerae* filtrates according to Burnet and Stone (1947). Finally the periodate-virus-RDE treated cells (PVR-cells, Fazekas de St.Groth, 1949) were washed in two changes of citrate-saline, a change of dilute anti-RDE serum, and three changes of physiological saline, and packed tightly by centrifuging. Cells treated with periodate and RDE only (PR-cells) served as controls.

METHODS.

Determination of reducing power.

The reducing power was determined by the Hagedorn-Jensen micromethod after hydrolysis with 1·5 N HCl at 100° C. for 3½ hours in a sealed tube—conditions found to give maximal values with ovomucin. Reducing power is expressed in mg. per cent. as glucose.

Titration of inhibitory activity of ovomucin.

The standard technique of Burnet (1948) was applied in the slight modification described previously (Gottschalk and Lind, 1949*a*).

EXPERIMENTAL.

24·0 ml. of ovomucin dispersion and 4·5 ml. of packed PVR-cells, both pre-cooled to 0° C., were mixed, kept for 90 minutes at 0° C. with occasional shaking, and finally centrifuged in the cold. In the control experiment ovomucin was treated with PR-cells accordingly. The original ovomucin dispersion and aliquots of the two supernatant fluids were titrated for inhibitory activity; the remainder was concentrated *in vacuo* to about one-third of its volume, hydrolysed, neutralized and analysed for reducing power.

Data of three representative experiments are given in Table I.

TABLE I.—*Inhibitory Titre and Carbohydrate Content of Ovomucin after Treatment with Virus-coated Red Cells.*

Number of experiment.	Ovomucin treated with-	Residual.		Percentage recovery.	
		Inhibitory titre.	Reducing power (mg.% glucose).	Inhibitor.	Carbohydrate.
1	PVR-cells	<5	177	<0·2	94
	PR-cells (control)	3000	189	100	100
2	PVR-cells	15	148	0·4	89
	PR-cells (control)	3500	166	100	100
3	PVR-cells	30	118	1·2	93
	PR-cells (control)	2500	127	100	100

PVR-cells were coated with inactive LEE (B) virus in Experiment 1; with active MEL (A) virus in Experiment 2; and with inactive MEL (A) virus in Experiment 3.

PR-cells were not treated with virus; the inhibitory titre of ovomucin was not significantly changed after treatment with these cells.

It is evident from the results that a 99 per cent reduction in inhibitory activity of ovomucin coincides with not more than 10 per cent loss of its carbohydrate moiety.

As a check on the selective adsorption of the inhibitor on to PVR-cells experiments were carried out with ovomucin, the inhibitory power of which had been destroyed by pre-treatment with active influenza virus. In this instance no decrease in the carbohydrate content was effected by PVR-cells (Table II).

TABLE II.—*Carbohydrate Content of Enzymically Inactivated Ovomucin after Treatment with Virus-coated Red Cells.*

Ovomucin* treated with—	Residual reducing power (mg.% glucose).	Percentage recovery of carbohydrate.
PVR-cells	156	100
PR-cells (control)	156	100

* The inhibitory titre of ovomucin was reduced from 4000 to <5 by pre-treatment with active MEL virus for 5 hours at 37° C.

DISCUSSION.

From results so far obtained it may be concluded that ovomucin consists of the inhibitor proper, lysozyme and a non-inhibitory fraction which may or may not be a single component, a concept of inhomogeneity already put forward in two preceding papers (Gottschalk and Lind, 1949*a* and 1949*b*). The same picture of complexity was recently arrived at by Lanni, Sharp, Csáky and Beard (1950) from the investigation of ovomucin by independent methods.

The quantitative data would suggest that the inhibitory component represents approximately 5 to 10 per cent of the total ovomucin fraction. This estimate rests on the assumption that both the inhibitory and non-inhibitory components are proteins containing comparable amounts of carbohydrate. The mucoprotein nature of the substrate (inhibitory component) is indicated by the characterization as a carbohydrate-peptide of the compound released from the substrate upon viral enzyme action (Gottschalk and Lind, 1949*b*). Furthermore the urine inhibitor of Tamm and Horsfall (1950), obtained in this Institute (Gottschalk and Ada, 1950) in electrophoretically homogeneous form by a modification of the original procedure, is a typical mucoprotein with a carbohydrate content closely resembling that of ovomucin. The mucoprotein nature of the inert non-inhibitory component follows firstly from a comparison of the overall analysis of ovomucin and of the inhibitor. Again, the close structural relationship between the two components is demonstrated by the complete failure to separate them by the common physico-chemical fractionating techniques, as experienced by all workers in this field.

Whereas the carbohydrate content of the inert portion of ovomucin could be determined accurately, the nitrogen determination was impaired by the unavoidable leakage of haemoglobin from periodate-treated cells ; however, the haemolysis would account for but a small proportion of the nitrogen recovered, amounting to 95 per cent or more of the original. This lends support to the main argument by showing the high protein content of the non-inhibitory component.

SUMMARY.

It is shown by selective adsorption to virus-coated erythrocytes that the virus haemagglutinin inhibitor constitutes only 5 to 10 per cent of the total ovomucin fraction, the remainder consisting of a non-inhibitory muco-protein and lysozyme.

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THE EFFECT ON EXPERIMENTAL TUBERCULOSIS OF THE INTRAVENOUS INJECTION OF INSOLUBLE SUBSTANCES: EXPERIMENTS WITH CARBON.

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EVEN before the introduction of any chemotherapeutic agent active against bacteria, and when the possibility of obtaining such agents was still thought to be remote, it was realized that in the chemotherapy of tuberculosis particularly difficult problems had to be solved.

Since the work of, in particular, De Witt (1913, 1914*a* and *b*, 1918) and Lewis (1912, 1916, 1917) the possibility of treating tuberculosis with a specific chemotherapeutic substance capable of entering and being stored by macrophages and epithelioid cells appears to have been dismissed. Now that new antibiotics are being tested in the treatment of tuberculosis it seems worth while to reopen this method of approach and to apply it to the investigation of appropriate antibiotic substances.

Wells and Long (1932), in discussing the chemical basis of the therapeutics of tuberculosis, remarked: "If we wish to destroy the tubercle bacilli in the body by specific chemotherapeutic measures we must consider, not only what chemicals can kill the bacilli in the test tube but also which ones can penetrate the tubercle and pass through the wall of the phagocyte in which" [they are] "so often entrenched," and again "In the animal body . . . the bacilli are protected by living cells from attack by agents introduced into the body of the infected animal, and generally these cells are much less resistant to strange chemicals than are the hardy, wax-armoured tubercle bacilli."

"If the drug is to be a true chemotherapeutic agent in Ehrlich's sense, that is, a substance that kills bacteria and spares the host because it has a high selective affinity for the bacterial protoplasm and a low affinity for the protoplasm of the host, it must be able to penetrate the tubercle, the phagocytic cell if the bacilli are enclosed within cells, and the tubercle bacillus. . . ."

These views are held at the present time, and there are still many gaps in the knowledge of the behaviour of chemical substances in tubercles.

Most pathologists favour the view that the main protection of the body against invasion by tubercle bacilli lies in the phagocytic cell—the macrophage and monocyte. These cells readily ingest the bacilli and in some instances kill and slowly digest them. But their ability to kill the bacilli is limited, and often instead of protecting the body they act as vehicles by reason of their motility, and spread the organisms, and are themselves eventually killed by them. Although there is abundant evidence that tubercle bacilli can live and divide extracellularly, it can be shown in experimental tuberculosis that infecting bacilli are intracellular, at least at the beginning.

If the macrophages in and around a tubercle could be "fortified" by providing them with a constituent capable of inhibiting the growth of or, better still, killing the tubercle bacillus, it might be possible to influence the tuberculous reaction in favour of the host so that an infection which would otherwise spread would be arrested. One way of doing this would appear to be to induce the macrophages to ingest an antibacterial substance in particulate form. There is no doubt that tissue macrophages are capable of ingesting particles presented to them in the tissues and of segregating certain vital dyes introduced into the blood stream. These properties are shared with fixed phagocytic cells such as those in the liver and spleen.

The epithelioid cells which often contain tubercle bacilli probably arise from monocytes

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or macrophages through the action of some constituent of the bacilli. There is no conclusive evidence that epithelioid cells once formed can phagocytose bacteria or segregate dyes such as trypan blue. There is some evidence from the literature and from the experiments recorded below that foreign material introduced into macrophages is found in epithelioid cells derived from them. It seems possible that macrophages that have ingested tubercle bacilli become changed to epithelioid cells, while still retaining their content of bacilli.

Even if an effective concentration of foreign material cannot be introduced into the epithelioid cells, a " fence " of macrophages containing an antibacterial agent around the periphery of a growing tubercle might prevent its spread, for the bacilli appearing at the periphery might be ingested by " fortified " macrophages and thus be more readily destroyed. Provided that the " fence " could be formed and maintained, this might be sufficient to enable the body to overcome the bacilli ; one could visualize the gradual healing of the tubercle in much the same way as in a body overcoming infection naturally.

That particulate matter will emerge from the blood stream when the vessels are abnormal is well known. It has been shown for example by Burrows (1932), who illustrated very clearly that carbon injected intravenously congregates around an inflamed area produced subcutaneously by the injection of agar into a transplanted sarcoma in a rat, as well as in the liver, spleen, bone marrow, etc. Menkin (1940) also figures carbon particles which have escaped from blood vessels in acute inflammation. Whether particles will emerge from vessels around a growing tubercle and be taken up by macrophages is not clear but it seemed to be a possibility.

The desirability of pursuing this line of experiment was raised when Su (1948) isolated an antibiotic, micrococcin, from a species of *Micrococcus*. Micrococcin is only very slightly soluble in water, but it inhibits the growth of Gram-positive organisms including the tubercle bacillus *in vitro*. It seemed possible that during the early stage of a tuberculous infection it might be made to enter macrophages or even epithelioid cells in or around the tubercle and so help to retard the development of the disease.

Micrococcin, however, is difficult to prepare in quantity, and to trace its distribution in the tissues frozen sections must be examined by ultra-violet light, a method which does not give good histological results (Markham, Heatley, Sanders, and Florey, 1951). On account of these two difficulties it was decided to start by reinvestigating the fate of finely divided carbon injected intravenously in tuberculous rabbits. It was hoped that this would give a forecast of the distribution of micrococcin in growing tubercles.

The early experimental evidence that particles such as those of metal-protein colloids and egg albumin do not enter tubercles in the same way as most crystalloids was summarized by Wells and Long (1932). Since that summary was written, Rich (1946) has studied the behaviour of colloidal iron (saccharated iron oxide) in patients with miliary tuberculosis, and has found that it does not penetrate into the substance of the tubercle, though it can be found in the phagocytes of the spleen, liver and bone marrow.

Recorded experiments on the behaviour of intravenously injected carbon and other particles in experimental tuberculosis were designed either to test the effect of carbon on the progress of the tuberculous lesion (Haythorn, 1913 ; Corper and associates, 1927, 1923), or to investigate the origin of the epithelioid cells (Foot, 1920, 1921 ; Westhues, 1922). As far as can be ascertained, no experiments have been made expressly to determine the phagocytic power of epithelioid cells, although Jaffé (1926) drew conclusions about this from his studies.

It may be stated very briefly that Haythorn, Corper *et al.*, Foot, and Jaffé all agree that in certain circumstances and in some organs carbon introduced into the blood stream finds its way into the macrophages surrounding developing tubercles and may be found in the epithelioid cells. Carbon is absent from pneumonic areas in the lung and is scanty in lesions developing in the medulla of the kidney. Westhues (1922) and Schlecht (1907) found that carmine particles were absent from epithelioid cells of well-developed tubercles of the lungs, the animals having been given lithium carmine before infection.

Jaffé's conclusion is of interest in connection with the present work. He stated that the ink found in tubercles had not been actively taken up by the epithelioid cells of the tubercle but had been transmitted to them by division of carbon-filled parent cells. He did not believe that the epithelioid cells had lost the power of phagocytosis, but that owing to the avascularity of the tubercle their environment had insufficient concentration of ink to enable them to take it up.

In spite of these previous investigations, not all the information required about the distribution in growing tubercles of carbon introduced intravenously was available.

EXPERIMENTAL.

The experiments now to be described aimed at ascertaining the distribution of small particles of carbon introduced into the blood stream of animals developing experimental tuberculous lesions, particular attention being paid to determining whether the distribution in the tubercle depended on the stage of its development at which carbon was first given.

Methods.

Ten rabbits were each inoculated by an ear vein with 0·01 mg. of a virulent strain of bovine tubercle bacilli. Daily injections of carbon were begun 4 days after inoculation in the first rabbit, and subsequently at 2-day intervals in the other animals. Two control animals received carbon only and bacilli only respectively.

The carbon suspension consisted of one part of a preparation of India ink (Gurr) diluted with three parts of distilled water, filtered once through a double thickness of filter paper and boiled. The resulting suspension consisted of dispersed particles not greater than 3μ in diameter and mostly considerably less. The particles were not agglutinated *in vitro* by horse or rabbit serum. Eight ml. of the suspension were given into an ear vein at each injection. Ten such injections were given to each rabbit, and in a control animal had no ill effect. During the later periods of the tuberculous infection, however, rabbits became obviously much more sick as the result of the carbon injections, and two died before the full number could be given. In this connection Scheinin and Peissachowitsch (1930) observed that animals inoculated with tubercle bacilli after the reticulo-endothelial system had been loaded with vital dyes died sooner than undyed animals.

Results.

The animals died, or were killed, between the 11th and 63rd days after infection. All except the one dying on the 11th day had fairly extensive gross lesions in the lungs and spleen, and usually in the liver and kidneys.

Macroscopic appearances.

The spleens were enlarged and contained many white nodules. The livers were always very black, sometimes with small white nodules. The lungs showed some variation in degree of blackness, being less dark the longer the period of survival. In two animals which died on the 19th and 27th days after the last carbon injection the lungs had a greyish colour quite distinct from the black lungs of animals which died soon after receiving carbon. The tuberculous areas stood out plainly as white plaques surrounded by a halo of tissue darker than the intervening lung. The impression that this halo represented an area of carbon concentration was not confirmed by histological examination, and it was probably due to greater cellularity about the zone of caseation. The bronchial glands were usually extensively involved.

The kidneys usually contained macroscopic lesions, and glomeruli could be seen standing out as black points in the greyish cortex.

Histological observations.

Portions of lungs, spleen, liver and kidney were fixed in mercuric formol solution, sectioned and stained by (1) haematoxylin and eosin, (2) the Ziehl-Neelsen method, using haematoxylin and metanyl yellow as counterstains.

The extent of the tuberculous lesions.—The rabbit that died on the 11th day had very early lesions in the intrapulmonary lymphoid tissue and small collections of epithelioid cells in the spleen. The one that died on the 21st day after infection had well-developed lesions in the lungs, spleen and liver, and smaller lesions in the kidney. As far as can be deduced from two animals, it would appear that the lesions developed fairly rapidly after the 11th day. All the other rabbits died later, and had extensive lesions in the lungs, spleen and liver and to a less extent in the kidneys.

In the lungs the largest lesions appeared to be in peribronchial lymphoid

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EXPLANATION OF PLATES.

FIG. 1.—The edge of a tuberculous lesion in the lung of a rabbit which had been injected intravenously with India ink. The injections began 18 days after infection. Note the fine dusting of carbon particles present in the epithelioid cells. Haematoxylin and eosin. × 640.

FIG. 2.—A lung lesion from a rabbit which had received 10 intravenous injections of carbon. The first injection was given 4 days after infection. Note that carbon is present in the centre of the lesion. Eosin only. × 60.

FIG. 3.—Rabbit lung. The animal was first injected with carbon 4 days, and was killed 40 days, after infection. × 300.

FIG. 4.—A tuberculous lesion in the lung of a rabbit which had received 10 intravenous injections of carbon. The first injection was given 20 days after infection with tuberculosis. Note the absence of carbon from the central area of the lesion, the scattering of particles in the peripheral epithelioid cells and the aggregates of particles in macrophages situated round the lesion. Eosin only. × 60.

FIG. 5.—A tubercle in the parenchyma of the liver of a rabbit which had been injected with carbon 20 days after infection. Ten intravenous injections of carbon were given. The caseous central area is free from carbon and the peripheral epithelioid cells contain more carbon than do the central cells. Haematoxylin and eosin. × 65.

FIG. 6.—The edge of a liver tubercle. Liver tissue can be seen in the right lower corner. The aggregates of carbon particles are becoming scattered in the tuberculous cells. Haematoxylin and eosin. × 640.

FIG. 7.—A giant cell, containing a large carbon "ring," in a tubercle in the liver of a rabbit. Haematoxylin and eosin. × 635.

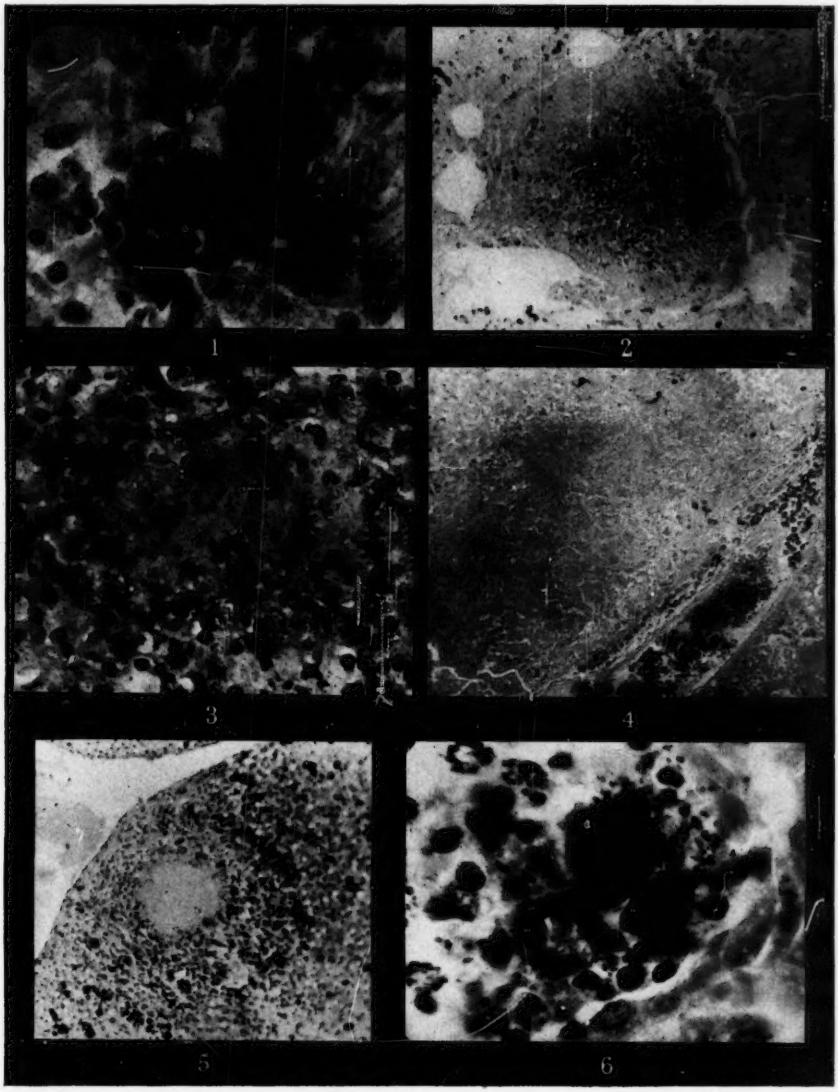
FIG. 8.—A tubercle which has grown in the portal tract of the liver. As the lesion has expanded into the liver tissue the edge away from the portal tract has taken up carbon from the Kupffer cells. Haematoxylin and eosin. × 64.

FIG. 9.—A lesion which has grown in a Malpighian body of the spleen. The lesion has spread into the surrounding pulp where the peripheral epithelioid cells can be seen "taking up" carbon. The redistribution of carbon with ring formation is well seen in the upper middle part of the field. This rabbit died 21 days after infection, the first carbon injection having been given 10 days after infection. Haematoxylin and eosin. × 68.

FIG. 10.—Cells at the periphery of a tuberculous lesion in the spleen. A redistribution of carbon is occurring. Note the characteristic ring formation. Haematoxylin and eosin. × 650.

FIG. 11.—A glomerulus in the kidney of a rabbit which died 40 days after infection. Carbon injections were begun on the 4th day. The greater part of the glomerulus has been replaced by tuberculous tissue through which carbon from the glomerular capillaries has become scattered. A carbon thrombus can still be seen in a surviving capillary on the left. Haematoxylin and eosin. × 300.

FIG. 12.—A tuberculous lesion in the kidney of a rabbit which died 63 days after infection. Carbon-containing glomeruli have become surrounded by a mass of epithelioid cells. Haematoxylin and eosin. × 60.



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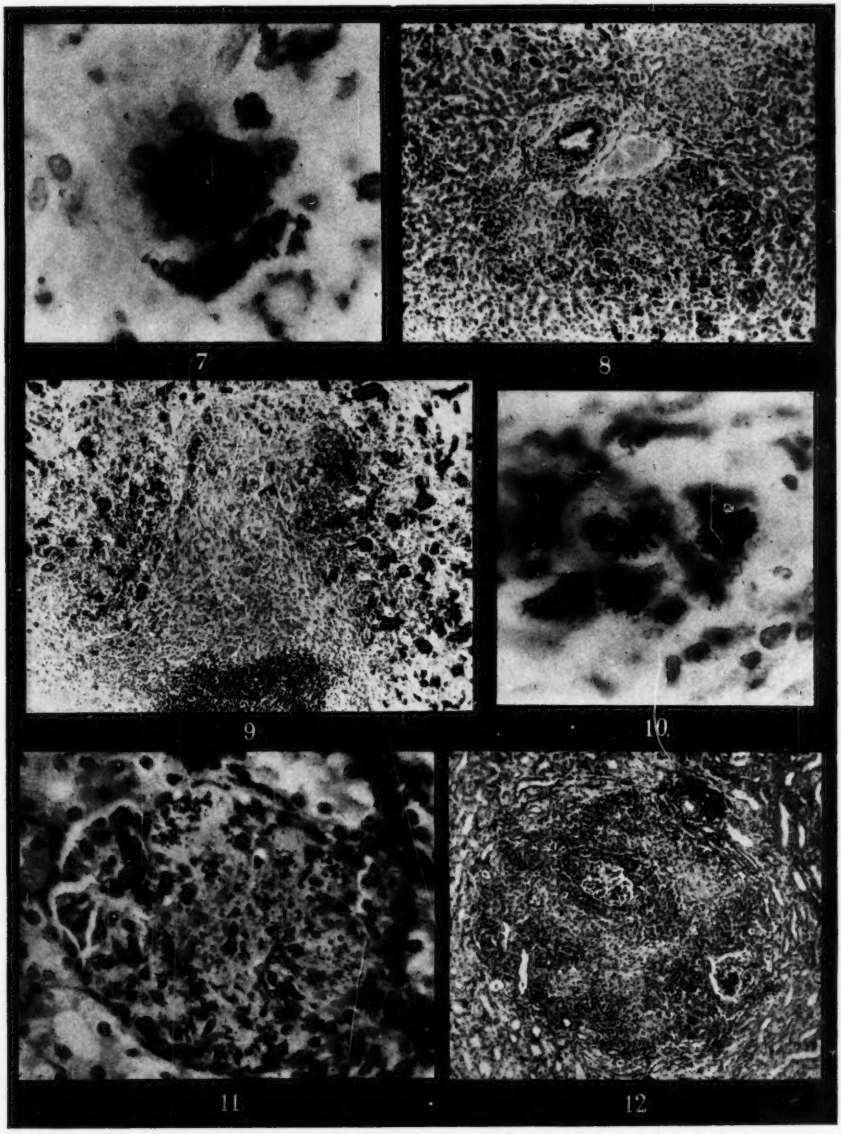

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tissue, but lesions of various sizes were scattered widely throughout the lungs, and in some of these extensive areas of caseation were present.

In every animal the spleen was the organ most heavily infected. Often it was completely disorganized. Many early lesions could be seen developing in or near splenic nodules (Zeyland, 1935, noted that this was the site of development of many splenic lesions). Caseation was more evident in the spleen than in any other organ.

In the liver the lesions varied from a single giant cell surrounded by a narrow zone of epithelioid cells to large caseous areas. Some were developing in the hepatic lobules, but most were in the periportal tissue.

Only a small number of lesions could be found in the kidneys, and most of these were situated in or near the glomeruli.

The distribution of carbon in tuberculous and non-tuberculous tissue. Lungs.—As noted macroscopically, carbon tended to disappear from the lungs with the passage of time, but even 36 days after the last carbon injection considerable amounts were still present. It was segregated in macrophages which were lying in the alveolar walls and which were widely scattered throughout the lung. These carbon-filled cells were sometimes plentiful about blood vessels, and sometimes thrombi containing densely packed, carbon-filled cells adhered to vessel walls. Tuberculous tissue often invaded these thrombi. Occasionally a vessel appeared to have been completely plugged with carbon which had later been phagocytosed. The remains of such blocked vessels were sometimes surrounded by tuberculous tissue into which the carbon had spread. Groups of carbon-filled cells sometimes formed plaques on the walls of vessels; these cells were probably monocytes which had phagocytosed a thrombus. The association between tuberculous tissue and thrombi was so frequent that probably the damage to the endothelium resulting from the tuberculous process was the primary cause of the thrombosis. Small carbon emboli were seen in a few capillaries.

Carbon was widely distributed throughout the tuberculous tissue of the lung, partly as large black masses situated in single cells or groups of cells, but mainly as a fine dusting of intracellular particles in the epithelioid cells (Fig. 1). Animals to which carbon had been given early often had tubercle bacilli and carbon particles in the same epithelioid cells. The cells containing the largest clumps of carbon were situated at the periphery of the tubercles and appeared to be pre-existing macrophages that had become engulfed in spreading tuberculous tissue. There was evidence that the load of carbon from these cells was becoming dispersed amongst surrounding cells. Thus in animals receiving carbon early in the infection the caseous areas and central cells were well filled (Figs. 2 and 3), but in those given carbon late the caseous centres contained little or no carbon, and the concentration increased from the central to the peripheral epithelioid cells (Fig. 4). In pneumonic areas where the alveolar walls were intact the mononuclear cells of the exudate were carbon-free, but where the structure of the lung had been destroyed, dispersion of macrophage carbon seemed to have taken place.

Liver.—The hepatic cells contained a fine dusting of carbon, while many of the Kupffer cells contained very large masses of particles. Evans, Bowman, and Winternitz (1914) found that the liver was the most suitable organ in which to follow the histogenesis of the miliary tubercle, and in the present work it was the easiest place in which to follow the distribution of carbon in the tubercle.

Tubercles in the liver lobules varied greatly in size, so that the distribution of carbon could be observed in both young and old lesions in one animal. Many tubercles were present in the periportal tissue. There were differences in the distribution of carbon in the lobular and portal lesions.

In lobular lesions in rabbits receiving carbon early in the infection the caseous areas and most of the epithelioid cells contained a liberal sprinkling of carbon, and carbon and bacilli were often seen in the same cell (an observation also made by Oeller, 1922). In rabbits that had been injected at a later stage the caseous central areas were carbon-free and the inner cells contained rather less carbon than the peripheral cells (Fig. 5). As the tubercle expanded and encroached more and more on the lobule, carbon-filled Kupffer cells became included in the tuberculous tissue and underwent structural changes there. The carbon, which was previously present in globular masses, became dispersed in the cell cytoplasm as fine granules, which sometimes had a ring formation (Fig. 6). Similar observations have been made by Sabin (Sabin, Cunningham, Sugiyama, and Kindwall, 1925; Sabin, Doan, and Cunningham, 1925; Sabin, 1932). At the same time some of the cells showed an increase in the number of nuclei (Fig. 7).

In the lesions in the portal tracts no carbon was present as long as the lesion remained confined to the tract. When it invaded a liver lobule the epithelioid cells adjacent to the lobule contained large amounts of carbon, and the Kupffer cells showed the structural changes described above. In such lesions, therefore, the portion situated in the portal tract was carbon-free while that in the liver lobule contained much carbon (Fig. 8).

Spleen.—The spleens were heavily packed with carbon and their architecture distorted by large areas of tuberculous tissue and caseation.

In animals receiving carbon late in the infection the caseous areas contained little or no carbon, and the inner contained less than the outer cells.

In several spleens tuberculous tissue developing in the nodes was still surrounded by lymphoid tissue; such lesions were always free from carbon. Lesions that had expanded beyond the lymphoid tissue had considerable amounts of carbon in the portion situated within the pulp (Fig. 9). The carbon-filled macrophages at the borders of such a lesion showed the same "ring" and giant cell modifications as were observed in the Kupffer cells of the liver (Fig. 10). Carbon distribution at the periphery of the portal tract lesions and of the lesions of the splenic nodules was very similar.

Kidney.—The glomeruli of the kidney were well filled with carbon, most of which seemed to be intracellular.

Early tuberculous lesions were illustrated very beautifully in many of these kidney sections. In most cases they consisted of a small group of epithelioid cells situated in part of a glomerular tuft. Older lesions pushed the remains of the tuft to one side (Fig. 11). and at a still later stage the glomerulus disappeared, leaving its carbon dispersed among the epithelioid cells. In larger lesions carbon-containing glomeruli were engulfed in a mass of epithelioid cells (Fig. 12). The ultimate destruction of these engulfed glomeruli would account for the presence of circumscribed areas of carbon-filled cells in a lesion which otherwise contained little carbon.

The occasional tubercles in the medulla were remarkable because only a rare cell contained any carbon at all; these were related to blood vessels and were

probably monocytes from the blood. The surrounding medullary tissue did not contain carbon except for an occasional cell associated with a vessel.

DISCUSSION.

A common explanation can be advanced to explain the distribution of carbon observed in tuberculous lesions in the liver, spleen and kidney, and in many lung lesions. It seems that tuberculous tissue spreads and comes into contact at its periphery with cells containing carbon, for instance Kupffer cells in the liver, the carbon then becoming re-distributed among the peripheral epithelioid cells. This could happen in two ways: either the carbon-containing macrophages could be killed by the tuberculous tissue and their carbon phagocytosed by the epithelioid cells, or, more probably, the macrophages could themselves be transformed into epithelioid cells under the influence of chemical substances from destroyed tubercle bacilli (Rich, 1946). The epithelioid cells by division then dilute and re-distribute the carbon that they contain.

Many of the appearances seen in the peripheral cells—the breaking up of carbon aggregates, the dispersion of carbon particles throughout the cell cytoplasm, the appearance of carbon rings—all seem to indicate that some drastic change takes place in the cytoplasmic arrangement of the cells. One is reminded of the changes observed by Sabin, Doan, and Forkner (1930) and Sabin (1932) to take place in monocytes and "clasmatocytes" under the influence of tuberculous products. In lesions of the lung the carbon was usually patchily distributed, whereas if the epithelioid cells phagocytosed carbon the distribution throughout the lesions should be fairly regular.

The wide and fairly regular distribution of carbon in the lesions when carbon was started soon after infection does not seem surprising. It will be recalled that lesions were only just beginning to appear 11 days after infection. The animals which had the most widely dispersed carbon in their lesions had started injections before this time. Thus, most of the reticulo-endothelial cells would already contain carbon before they phagocytosed tubercle bacilli. As these cells became transformed into epithelioid cells and proliferated, the carbon would become evenly distributed in the young and growing tuberculous tissue. In the animals which were first given carbon 20 days after infection there were relatively isolated groups of carbon-containing cells at the periphery of the lesions. The involvement of carbon-containing tissue macrophages in the spreading edges of lesions already formed would seem to explain this patchy distribution. So, too, would it explain the fact that the tubercles containing the most carbon were situated in tissues where carbon-containing cells were plentiful. The dependence of the tubercle for its content of carbon on the tissue in which it is growing was well shown by lesions starting in the portal tracts of the liver or the germinal nodes of the spleen, both areas almost entirely free of carbon. Only those parts of the tubercles which spread into adjoining liver parenchyma or spleen pulp contained any significant amount of carbon. It would thus seem that few carbon particles actually emerge from the blood vessels in the neighbourhood of growing tubercles, though this clearly does not exclude the possibility that smaller or colloidal particles may do so.

There remains one further aspect of the carbon distribution to be explained. This is the presence of a few carbon-containing cells within a lesion growing in a

carbon-free tissue such as the medulla of the kidney. In an experiment not recorded here the same appearance was observed in a very chronic lesion in the kidney of a rabbit that had been given carbon intravenously 120 days after inoculation with human tubercle bacilli. The cells containing carbon were few and widely separated, and most of them were lying near capillaries. In some the carbon was clumped and not dispersed in small particles as it usually is in epithelioid cells. There seems strong reason for believing that these cells are carbon-containing monocytes or perivascular macrophages which have wandered from the vessels into the tuberculous tissue.

In general, this work indicates that carbon will only penetrate throughout quite young (or at least quite cellular) lesions ; that there is no special tendency for epithelioid cells to take up carbon, the appearances suggesting that they " inherit " rather than " acquire " it ; and that the amount of carbon in a lesion is governed by the amount in the tissue that the lesion replaces. The extent to which the vascularity of a lesion determines the distribution of carbon within it has yet to be studied.

These experiments showed that in certain circumstances particles could be made to penetrate into both the macrophages and epithelioid cells of tubercles and that this result was intimately dependent on the time at which the carbon was given.

SUMMARY AND CONCLUSIONS.

From the present work and from that of previous authors, many of whose observations have been confirmed, it is concluded that :

Carbon given intravenously to rabbits infected with bovine tubercle bacilli is most widely distributed through the tubercles if it is given within ten days after infection.

Carbon is most plentiful in tubercles situated in tissues which store carbon (e.g. parenchyma of liver and pulp of spleen) and about vessels containing carbon thrombi. Tubercles, or parts of tubercles, growing in tissue where carbon is not stored tend to be carbon-free.

If carbon is given very early in the infection most of the cells which go to form the early lesions in, e.g., the liver and spleen, contain carbon before they take up tubercle bacilli ; in such tubercles the carbon in the lesion is plentiful and diffuse, and carbon and tubercle bacilli are frequently observed in the same cell.

When carbon is given some time after inoculation the periphery of the spreading tuberculous tissue engulfs carbon-filled tissue macrophages.

The epithelioid cells do not selectively take up carbon, and generally contain less than cells in the surrounding areas. The carbon is finely dispersed within the cell, in contrast with the heavy aggregates seen in macrophages. It is possible that carbon-filled macrophages are transformed into epithelioid cells, when intracellular re-distribution of the carbon takes place, followed by dispersion among daughter cells as the epithelioid cells divide.

In rapidly spreading tuberculous tissue, and in pneumonic areas in the lung, carbon is absent from the epithelioid cells.

A few carbon-containing monocytes or macrophages seem to be able to leave vessels or their neighbourhood and infiltrate into tuberculous tissue without undergoing any apparent change of form.

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THE INACTIVATION OF THE BLOOD GROUP RECEPTORS ON THE HUMAN ERYTHROCYTE SURFACE BY THE PERIODATE ION.

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THE rapidity with which dilute solutions of sodium periodate inactivate the water-soluble human blood group mucoids (Morgan, 1947*a* ; Aminoff and Morgan, 1949, 1951 ; Annison and Morgan, unpublished work) suggested that the examination of the action of this reagent on the intact human erythrocyte might yield some interesting information as to the relative susceptibility of the group receptors present, and perhaps give some indication of the nature of those blood group factors which do not normally appear in water-soluble form—for example, the materials associated with M, N, P and Rh specificity.

The use of periodate in virus receptor studies has demonstrated that treatment of red cells with the reagent does not result in extensive morphological damage to the cells. Thus Hirst (1948) examined the influence of a number of oxidizing agents on fowl red cells, and reported that, although sodium periodate in 0·001 M concentration rapidly abolished virus receptor activity and led to changes in haemoglobin colour, the morphology of the erythrocytes was unchanged.

An account of the action of potassium periodate on the human red cell was given by Stewart (1949), who showed that the specificity of the cell was changed in such a way that the treated cells were capable of stimulating the production of specific antibodies in the rabbit which did not react with untreated cells. Stewart also found that the erythrocytes after treatment with periodate were rendered panagglutinable by normal human sera. The behaviour of the specific blood group characters of the cells, however, was not examined. Moskowitz and Treffers (1950) confirmed the presence of an agglutinin in normal sera for red cells which had been treated with periodate, and reported that the A and B blood group receptors were not destroyed by treatment with 0·001 M potassium periodate at pH 7 for 30 minutes at room temperature. Under these conditions the *Rhesus* character (as measured by the D antigen) was apparently destroyed completely, but no details of the serological tests were given.

The results of a systematic examination of the action of periodate ions, at different concentrations and pH values and for different times, on the serological behaviour of the human blood group characters A, B, O, H, Le^a, *Rhesus* (D), M, N and P are reported in this paper.

EXPERIMENTAL.

Materials and Methods.

Periodate solutions.

Dilutions in normal saline were made from a stock 0·5 M-HIO_4 solution.

Buffer solutions.

Acetate buffer (M) was used for the range pH 4·0–5·0. For pH 7·0 Na_2HPO_4-KH_2PO_4 buffer (0·66 M) was used.

Treatment of red cells with periodate.

Red cells were treated with 0·005 M and 0·001 M periodate at pH 7·0, 5·0, 4·5 and 4·0. Freshly drawn citrated blood was centrifuged and the cells washed several times with normal saline. Suspensions (10 per cent) in saline were prepared from the packed cells, and 2 volumes were mixed with 1 volume of buffer solution and 1 volume of periodate solution. The cell suspensions were kept at $15 \pm 1°$ with frequent but gentle mixing. Samples were withdrawn at intervals, diluted immediately with an equal volume of glucose-citrate solution (30 g. sodium citrate, 25 g. glucose to 1000 ml. water) in order to prevent further reaction of the periodate, centrifuged, and the deposited cells washed once with glucose-citrate solution. For use in the agglutination tests 1 per cent suspensions in glucose-citrate solution were prepared from the washed cells, and were then filtered through plugs of cotton-wool to remove any clumps of cells. Treatment with periodate at pH 4·0, 4·5 and 5·0 caused the cells to become brown in colour, but they appeared normal in shape, or at most only slightly distorted, and the suspensions were free from haemolysis. The rapidity with which the cells changed colour increased with increase in the acidity of the buffer and with the strength of periodate used. Red cells suspended in buffer solutions at these pH values, in the absence of periodate, did not change colour, and after centrifugation and washing gave suspensions in glucose-citrate solution which showed normal stability over a period of several hours. Treatment of red cells with 0·001 M periodate at pH 7·0 up to a period of 2 hours did not give rise to visible change in the cells, but exposure to 0·005 M periodate at this pH resulted in extensive haemolysis; red cells treated under these conditions could not therefore be tested for loss of blood group activity.

Test sera.

Human sera were used for A, B, O, P, Le^a and *Rhesus* (D) tests (Schiff and Boyd, 1942; Wiener, 1943; Race and Sanger, 1950). Owing to the presence of an agglutinin in all human sera for cells treated with periodate (Stewart, 1949; Moskowitz and Treffers, 1950) it was necessary to absorb these sera before use with periodate-treated cells which did not contain the blood group receptor under investigation. For example, in order to test the activity of A cells treated with periodate the anti-A serum used was previously absorbed with O cells treated with periodate. Sera were absorbed for 20 minutes at 10° with 0·1 volume of red cells which had been exposed to 0·005 M periodate at pH 5·0 for 30 minutes. The absorption was repeated until there was no appreciable agglutination of the absorbing cells by the undiluted serum. The H, M and N activities of cells were tested by means of immune rabbit sera. These sera did not contain agglutinins for cells treated with periodate and therefore absorption was unnecessary.

Agglutination tests.

The method consisted in each instance of adding a constant volume of cell suspension (1 per cent) to serial dilutions of serum, and leaving the mixture to

stand 2 hours at the appropriate temperature, after which time the tubes were examined for agglutination. The agglutination end-point was taken as the last dilution at which clumps of two or three cells were visible under the low power of the microscope. Differences in the strength of agglutination, the amount of serum available and the optimum temperature for the reaction necessitated slight variations in the technique employed in testing for the different factors. A and B tests were carried out by mixing 0·1 ml. of cell suspension, accurately measured, with an equal volume of serum dilution in tubes measuring 0·5 in. by 2 in. Owing to the limited amount of anti-M, N, H, O, Le^a, P and *Rhesus* (D) sera available, when tests were carried out with these reagents the serum dilutions and cell suspensions were measured into small tubes (0·25 in. by 2 in.) by means of a Pasteur pipette graduated at 0·02 ml. Throughout the reaction period A, B, M and N tests were kept at room temperature, H, O, Le^a and P at 12–14°, and *Rhesus* (D) cells were incubated with the serum at 37°. The tubes containing A, B, M and N cells and the corresponding agglutinins were given three standard taps before reading, whereas, in view of the ease with which the agglutinated cell clumps are broken up, H, O, Le^a, P and *Rhesus* (D) serum-erythrocyte suspensions were transferred gently and without shaking to the microscope slide and spread for observation as a thin film.

Absorption tests.

In addition to agglutination tests with the red cells after treatment with periodate, the destruction of the specific blood group receptors was measured in some instances by following the decrease in the capacity of the cells to absorb antibody from the homologous serum. The volume of packed untreated cells of the appropriate group which it is necessary to add in order to absorb the specific antibody almost completely from a given volume of serum was determined for each serological character in a preliminary experiment. Suspensions (10 per cent) in glucose-citrate solution were prepared from red cells treated with periodate as described above, and a sample containing the previously determined volume of cells was measured into a centrifuge tube. The suspension was centrifuged, the supernatant removed and the given amount of serum was added to the deposited cells. The mixture was stirred with a glass rod to ensure an even suspension, and was then kept at 8–10° for 30 minutes with frequent shaking. The agglutination titre of the absorbed serum with untreated cells of the appropriate group was then determined.

Precipitation tests.

The action of periodate on protein solutions was followed by means of serological precipitation tests. These tests were made by mixing 0·1 ml. of undiluted test-serum with 0·1 ml. of serial dilutions of the treated protein solution in normal saline. After 2 hours at 37° the tubes were kept overnight at 0° and examined while still cold by means of a hand lens.

Treatment of protein solutions with periodate.

Protein solutions diluted 1 : 500 (2 ml.) were added to 0·02 M HIO_4 solution (1 ml.) and buffer solution pH 5·0 (1 ml.) and the mixtures were allowed to stand at 15°. Samples were removed after 15, 45 and 120 minutes and diluted with

an equal volume of 0·05 M-Na_2HPO_4 in glucose-citrate solution. Addition of this latter solution served to bring the pH to 7 and to remove excess periodate. The solutions were then centrifuged and tested for precipitation with the homologous antiserum.

Coombs test.

The indirect test described by Coombs, Mourant and Race (1945) for the detection of " non-agglutinating " *Rhesus* antibodies was used in order to obtain red cells with a surface coating of protein which could be detected by means of agglutination tests. Human group O homozygous *Rhesus* positive (DD) cells (20 per cent) suspended in normal saline were added to an equal volume of " non-agglutinating " anti-D serum, and the mixture was incubated at 37° for 30 minutes. The suspension was then centrifuged and the cells were washed twice with saline. A 1 per cent suspension in saline was prepared from the washed cells and was added to serial dilutions of rabbit anti-human-γ-globulin serum. Agglutination of the treated cells was thus an indication that combination with the " non-agglutinating " antibody (γ-globulin) had occurred. *Rhesus*-negative cells of group O which had been similarly treated with " non-agglutinating " anti-D serum, and *Rhesus*-positive (DD) of the same group cells which had not undergone previous treatment, were included as controls in the agglutination tests with the anti-γ-globulin serum.

Preparation of stromatin.

Stromatin from human red cells was prepared by the method of Calvin, Evans, Behrendt and Calvin (1946). Group A cells were washed twice with saline, cooled to 0°, and then haemolysed by pouring into 10 volumes of water at 0–4°. N/1 HCl was cautiously added with thorough stirring until the pH was reduced to 5·7. The precipitated stromata were rapidly collected in a Sharples centrifuge, and were then washed alternately with dilute phosphate buffer pH 5·0 and distilled water until the supernatant was free from haemoglobin. The stromata were resuspended at pH 7·6, and centrifuged at 50,000 G. Stromatin was obtained from the clear supernatant fluid by readjustment of the pH to 5 with dilute acetic acid. The precipitate was centrifuged, suspended in saline at 0°, and the pH brought to about 7·3 with dilute alkali. The resultant clear solution was stored at 2–3° in the presence of 1 : 1000 merthiolate.

Preparation of anti-stromatin serum.

Three rabbits were given 4 intravenous injections of stromatin (1 mg., 2 mg., 5 mg., 5 mg.) spaced at 2–3 day intervals. Serum samples, taken 8 days after the last injection, gave precipitates with the injected antigen.

RESULTS.

(1) *The Influence of Periodate on the Specific Blood Group Receptors on the Erythrocyte Surface.*

Treatment of human erythrocytes with periodate resulted in loss of activity of all the blood group factors examined, but it was possible by varying the concentration of periodate used and the pH at which the reaction was carried out to

detect differences in the relative susceptibilities of the various specific receptors to the action of the oxidizing agent.

(a) *The A_1 and B receptors.*

The results of a typical inactivation experiment showing the fall in A specific activity after exposure of A_1 cells to two concentrations of periodate for various time intervals, and at different pH values, are given in Table I. Group O cells

TABLE I.—*Inactivation of A Structures on the Erythrocyte Surface by Periodate.*

Cell group.	Treatment. pH.	IO_4' (M conc.).	Time (min.).	Dilution of anti-A test serum. 1 : 4.	1 : 8.	1 : 16.	1 : 32.	1 : 64.	1 : 128.	1 : 256.
A	4·0	—	15	3	3	3	3	2	1	tr.
„	„	0·001	„	1	tr.	0	0	0	0	0
„	„	0·005	„	tr.	0	0	0	0	0	0
O	„	„	„	tr.	0	0	0	0	0	0
A	4·5	—	15	3	3	3	3	2	1	tr.
„	„	0·001	„	2	2	1	tr.	0	0	0
„	„	0·005	„	1	tr.	0	0	0	0	0
O	„	„	„	tr.	0	0	0	0	0	0
A	5·0	—	120	3	3	3	3	2	1	0
„	„	0·001	15	3	3	3	3	2	1	0
„	„	„	45	3	3	3	2	1	tr.	0
„	„	„	120	3	2	2	1	0	0	0
O	„	„	„	1	0	0	0	0	0	0
A	„	0·005	15	3	3	3	2	1	0	0
„	„	„	45	2	1	0	0	0	0	0
„	„	„	120	1	tr.	0	0	0	0	0
O	„	„	„	tr.	0	0	0	0	0	0
A	7·0	—	„	3	3	3	3	2	1	tr.
„	„	0·001	15	3	3	3	2	2	1	tr.
„	„	„	45	3	3	3	2	2	1	0
„	„	„	120	3	3	3	2	2	1	0
O	„	„	„	1	0	0	0	0	0	0

Degrees of agglutination: tr., trace of agglutination; 1, groups of 2–3 cells; 2, larger groups with many free cells; 3, agglutination visible to the naked eye.

were also treated with periodate under the appropriate conditions and included in each test to measure the amount of agglutinin remaining in the absorbed test-serum for cells treated with periodate. Under the most drastic conditions used (0·005 M HIO_4 at pH 4·0) the agglutination end-point with A_1 cells fell from 1 : 256 to 1 : 4 after exposure of the cells for 15 minutes, thus indicating that approximately 1 per cent only of the original activity remained. Treatment with more dilute periodate (0·001 M) at this pH again resulted in extensive inactivation, and a reduction of five tubes in the agglutination end-point was observed. At pH 5·0 the more dilute periodate was less effective, and treatment for two hours was necessary before a change measurable by the agglutination technique could be detected, but with 0·005 M periodate the activity had fallen to approximately 50 per cent of the original in 15 minutes and to about 6 per cent after 45 minutes. The fall of one tube in the agglutination end-point after exposure of the cells to 0·001 M periodate at pH 7·0 for 45 minutes was not increased after treatment for 2 hours, and this result suggests that very little inactivation of A receptors on the erythrocyte surface occurs in the presence of dilute periodate in neutral

solution. The course of inactivation of the B factor was essentially similar to that described for A ; the results are summarized in Table II.

In order to verify that the fall in the agglutination end-point observed after treatment of A_1 cells with periodate was due to inactivation of the specific A

TABLE II.—*Inactivation of A, B, H and O Structures on the Erythrocyte Surface by Periodate.*

Treatment of red cells.			Agglutination titre of cells with homologous antiserum.			
pH.	IO_4' (M conc.).	Time (min.).	A.	B.	O.	H.
4·0	—	15	256	512	256	1280
„	0·001	„	8	16	4	80
„	0·005	„	4	8	4	40
4·5	—	„	256	256	—	—
„	0·001	„	32	32	—	—
„	0·005	„	8	8	—	—
5·0	—	120	128	512	256	2560
„	0·001	15	128	512	256	2560
„	„	45	128	256	128	1280
„	„	120	32	128	16	320
„	0·005	15	64	256	32	1280
„	„	45	8	8	8	160
„	„	120	8	4	4	80
7·0	—	„	256	512	256	2560
„	0·001	15	256	512	256	1280
„	„	45	128	256	128	640
„	„	120	128	256	64	640
Control cells after treatment with IO_4'			O	4	4	—	—
			A_1B	—	—	4	40

receptors, and not to other changes in the red cells which prevented agglutination taking place, absorption experiments were carried out with the cells after treatment with periodate. Thus, if the A_1 receptors remained unchanged and combined with anti-A agglutinins, although agglutination failed to occur, the serum absorbed with the periodate-treated cells would show the same lowering of the agglutination end-point as would serum absorbed with untreated A_1 cells. The results of such an experiment given in Table III show that the absorptive power

TABLE III.—*The Agglutination Titre of Anti-A Serum with Untreated A_1 Cells after Absorption of the Serum with A_1 Cells Treated with Periodate.*

Cells used for absorption.				Agglutination titre of absorbed serum against untreated A_1 cells.
Group.	Treatment.			
	pH.	IO_4' (M conc.).	Time (min.).	
A	—	—	—	4
„	5·0	0·005	15	4
„	„	„	45	64
„	„	„	120	64
O	„	„	„	512
A	7·0	0·001	15	4
„	„	„	45	4
„	„	„	120	4
Titre of unabsorbed serum with A_1 cells				1024

of the A_1 cells diminishes after treatment with 0·005 M periodate at pH 5·0 for periods up to 2 hours, but exposure to 0·001 M periodate at pH 7·0 for this length of time did not give rise to a detectable decrease in the absorbing capacity of the cells. These results, therefore, confirm the findings obtained in simple agglutination tests with A_1 cells treated with periodate.

(b) *The O and H receptors.*

The differentiation of so-called anti-O sera into anti-O and anti-H, and the recognition of two distinct receptors, designated O and H, on the red-cell surface, are based on the reactions of a human anti-O serum examined by Boorman, Dodd and Gilbey (1948). These authors investigated the behaviour of 500 blood samples with this serum, and found that the results were in very close agreement with the frequencies which would be expected if the antibody were reacting with the product of the Bernstein O (and A_2) gene. A comparison of the reactivity of this serum with cells of different genotypes with the reactivity of the so-called anti-O sera from cattle, from goats immunized with *Sh. shigae* and from rabbits injected with purified " O-substance " prepared from human pseudo-mucinous ovarian cyst fluids (Morgan and Waddell, 1945), revealed discrepancies which could not be explained if the latter group of sera was also detecting the product of the O gene. It was proposed, therefore, that the factor detected by these sera should be called H, and that the symbol O should be reserved for the product of the Bernstein O gene ; the corresponding antibodies were therefore termed anti-H and anti-O (Boorman, Dodd and Gilbey, 1948 ; Morgan and Watkins, 1948). Examination of a number of human sera which agglutinate O cells has since revealed several examples of sera which behave similarly to the anti-O serum described by Boorman, Dodd and Gilbey (1948). In addition human sera are not infrequently encountered which are similar in reactivity to the animal anti-H sera.

The influence of periodate on the O and H receptors present in group O cells is shown in Table II, in which the agglutination titres with the homologous antisera before and after treatment of the cells with the oxidizing agent are recorded. On the whole, loss of O specific activity was slightly more rapid than loss of H activity, but the difference was not very marked. After treatment for 15 minutes with 0·001 M periodate at pH 4·0 a lowering of six tubes in the agglutination end-point of the treated cells with anti-O serum, and a reduction in the end-point of four tubes with anti-H serum, was observed. Exposure to 0·005 M periodate at this pH resulted in almost complete inactivation of both factors. At a rather less acid pH (5·0) both O and H receptors were relatively unchanged by treatment with 0·001 M periodate for periods up to 45 minutes, but after 2 hours the H activity fell to about 12 per cent and the O activity to 6 per cent of the original value. The greatest difference in the susceptibilities of the two factors was apparent after treatment with 0·005 M periodate at pH 5·0 for 15 minutes, when the agglutination end-point with anti-H serum was lowered by only 1 tube (about 50 per cent of original activity remaining), whereas the end-point with anti-O serum fell by four tubes (about 12 per cent of the original activity remaining). The O and H specific structures both appeared to be fairly resistant to the action of 0·001 M periodate at pH 7·0, and treatment of O cells for 2 hours resulted in a reduction of the agglutination end-point of two tubes only with both anti-O and anti-H sera.

(c) *The M and N receptors.*

The M and N receptors were found to be more susceptible to the action of periodate ions than any of the other factors which have been so far considered. The agglutination titres of M and N cells, belonging to group O, with the corresponding antisera after treatment of the cells with 0·001 M periodate at pH 5·0 and 7·0 are recorded in Table IV. After contact with the oxidizing agent for 15 minutes

TABLE IV.—*Inactivation of M, N, P,* Rhesus (*D*) *and Le*^{a} *Structures on the Erythrocyte Surface by Periodate.*

Treatment of red cells.			Agglutination titre of cells with homologous antiserum.				
pH.	IO_4'. (M conc.).	Time. (min.).	M.	N.	P.	*Rhesus* (D).	Lea.
4·0	—	15	—	—	32	—	32
,,	0·001	,,	—	—	4	—	8
,,	0·005	,,	—	—	2	—	4
5·0	—	120	32	64	64	512	64
,,	0·001	15	4	16	64	64	64
,,	,,	45	2	8	64	16	64
,,	,,	120	2	8	16	4	16
,,	0·005	15	—	—	64	16	32
,,	,,	45	—	—	32	4	8
,,	,,	120	—	—	16	4	8
7·0	—	,,	32	64	64	512	64
,,	0·001	15	4	16	64	64	64
,,	,,	45	2	8	64	32	64
,,	,,	120	2	8	32	16	32
Control cells after treatment with IO_4'	N		2	—	—	—	—
	M		—	8	—	—	—
	P-negative		—	—	4	—	—
	Rh-negative		—	—	—	4	—
	Le(a-)		—	—	—	—	2

at pH 5·0 group M cells retained about 6 per cent only (a reduction of 4 tubes in the agglutination end-point) of the activity of untreated M cells against anti-M serum, and after 2 hours at this pH inactivation was complete. At pH 7·0 the reaction took place more slowly, but a lowering in the end-point of two tubes was apparent after 15 minutes' treatment, and about 3 per cent only of the original reactivity remained after the periodate had been allowed to react with the cells for 2 hours. The anti-N test serum (titre 1 : 64), showed some reactivity with M cells (1 : 8) and it was possible therefore to detect only a 4-tube reduction in the agglutination end-point (12 per cent or less activity remaining). The results indicate, however, that inactivation of the N receptors was practically complete after 45 minutes' treatment with 0·001 M periodate at both pH 5·0 and 7·0.

The agglutination titres, obtained with untreated M cells, of samples of anti-M serum which had been absorbed under standard conditions with M cells treated with 0·001 M periodate for various lengths of time are shown in Table V. The failure of the cells to absorb significant amounts of antibody from the serum provides additional evidence that rapid loss of M specificity occurs in the presence of periodate ions.

TABLE V.—*The Agglutination Titre of Anti-M Serum with Untreated M Cells after Absorption of the Serum with M Cells Treated with Periodate.*

Cells used for absorption.				Agglutination titre of absorbed serum against untreated M cells.
Group.	Treatment.			
	pH.	IO_4' (M conc.).	Time (min.).	
M	5·0	—	120	2
„	„	0·001	15	32
„	„	„	45	32
„	„	„	120	32
N	„	„	„	32
M	7·0	—	„	2
„	„	0·001	15	16
„	„	„	45	32
„	„	„	120	32
N	„	„	„	32
Titre of unabsorbed serum with M cells				32

(d) *The P receptor.*

The results of the action of periodate on the agglutinability of P-positive cells belonging to group O with human anti-P serum are summarized in Table IV. At pH 4·0 the activity of the cells was reduced to about 12 per cent after treatment for 15 minutes with 0·001 M periodate, and to 6 per cent of the original value after treatment for the same length of time with 0·005 M periodate. The agglutination end-point of the cells was unchanged after treatment with the more dilute periodate (0·001 M) at pH 5·0 for 45 minutes, and had only fallen 2 tubes after the oxidizing agent had been allowed to react for 2 hours. In the presence of the higher concentration of periodate (0·005 M) at pH 5·0 the P-specific structures appeared to be more stable than the other blood group factors examined, and about 25 per cent of the original activity remained after 2 hours' treatment. In neutral solution 0·001 M periodate brought about a lowering in the agglutination end-point of 1 tube only after 2 hours, thus indicating that 50 per cent or more of the original activity remained unchanged. In all the tests described P-negative cells belonging to group O treated with periodate under the same conditions as the P-positive cells were included as controls.

Absorption experiments with P-positive cells which had been exposed to 0·001 M periodate at pH 7·0 and with cells treated with 0·005 M periodate at 5·0 confirmed the results obtained by means of the agglutination tests, and supported the conclusion that the P group receptor was one of the structures most resistant to the reagent.

(e) *The Le^a receptor.*

A summary of the results of the inactivation of Le^a structures on the erythrocyte surface by periodate is included in Table IV. The tests were carried out with group O, Le (a+) cells. Group O, Le (a—) cells treated under the appropriate conditions with periodate were also included in the agglutination tests as a measure of the residual agglutinin for cells treated with periodate remaining in the absorbed anti-Le^a serum. It will be observed that inactivation of the Le^a factor follows a course similar to that of the P receptors. The activity was reduced to about 25 per cent of the original value after treatment for 15 minutes

with 0·001 M periodate at pH 4·0, and to about 12 per cent with 0·005 M periodate. At pH 5·0 it was not possible to detect any loss of activity until after 2 hours' treatment with 0·001 M periodate, although more rapid inactivation occurred in the presence of more concentrated periodate (0·005 M). As had been found previously for A, B, H and P serological characters, loss of activity with 0·001 M periodate at pH 7·0 proceeded very slowly, and the agglutination end-point was lowered by only one tube after the reagent had been allowed to react with the cells for 2 hours.

(f) *The* Rhesus (*D*) *receptor.*

The influence of periodate on the *Rhesus* antigens on the erythrocyte surface was followed in the case of the D antigen by measuring the agglutination titre of the cells, before and after treatment, with an anti-D serum, and also by testing the capacity of the treated cells to absorb the antibody from anti-D serum. For the agglutination tests a potent anti-D serum (titre 1 : 512 in saline) was selected. Preliminary absorption experiments carried out with this serum, however, revealed that as many as five absorptions were necessary to cause a measurable lowering of the titre. This serum did not therefore appear to be suitable for absorption tests with the treated cells, and it was suggested to us by Dr. R. R. Race that the difficulty was due to the presence of considerable amounts of "non-agglutinating" D antibodies in the serum, which preferentially coated the cells and prevented their combination with, and the removal of, the antibody which acted in saline solution. In order to overcome this difficulty a number of less potent anti-D sera which agglutinated cells in saline were examined, and one was selected from which the antibody could be removed almost completely by one absorption with an equal volume of packed D cells.

The agglutination titres with anti-D serum of D cells treated with 0·001 M periodate at pH 5·0 and 7·0, and with 0·005 M periodate at 5·0, are given in Table IV. It is to be noted that inactivation of the D receptors occurred rapidly at both pH values. The agglutination end-point was lowered by five tubes after treatment of the cells for 45 minutes with 0·001 M periodate at pH 5·0, and with more concentrated periodate (0·005 M) for the same length of time at pH 5·0 the end-point fell to that given (1 : 4) by *Rhesus*-negative control cells which had been treated with periodate. It would therefore appear that under these conditions complete inactivation of the specific D structures occurs. At pH 7·0 treatment with 0·001 M periodate brought about a lowering of the agglutination end-point from 1 : 512 to 1 : 64 after 15 minutes and to 1 : 16 after 2 hours, thus indicating that about 3 per cent only of the original activity remained at the end of this time.

Absorption experiments with D cells treated with periodate confirmed the rapid inactivation of the D serological character which was observed when the treated cells were tested for agglutination. Absorption of the anti-D serum with untreated D cells reduced the agglutination end-point from 1 : 128 to 1 : 4, whereas after absorption with similar cells exposed to 0·001 M periodate at pH 7·0 for 15 minutes a one-tube reduction only in the end-point was obtained, and after more prolonged treatment with the oxidising agent the cells failed to remove sufficient antibody from the serum to cause any observable change in the agglutination titre.

(2) *The Influence of Periodate on Stromatin as Measured by the Specific Precipitation Reaction.*

The immunization of rabbits with stromatin was undertaken in the hope that agglutinins for a naturally occurring protein component of the human erythrocyte surface would thereby be produced. In the event of obtaining such antibodies it would thus be possible to investigate the influence of periodate on a component of the red cell membrane known to be protein in nature. The sera from the immunized rabbits reacted with the injected antigen in precipitation tests, but no increase in the agglutination titre was obtained with human red cells, other than those of group A from which the stromatin was prepared.

In order to determine whether the serological specificity of stromatin is destroyed by contact with periodate, a 0·1 per cent solution of the protein was treated at pH 5·0 with a 0·005 M solution of the oxidizing agent for various lengths of time, and was then tested for precipitation with rabbit anti-stromatin serum. The results showed that although there was a slight weakening in the degree of precipitation after treatment for 2 hours a considerable measure of the specificity of the native protein was retained.

(3) *Action of Periodate on Erythrocytes Sensitized with "Non-agglutinating" Antibody.*

In view of the failure to produce antibodies specific for a naturally occurring protein component of the erythrocyte surface, an attempt was made to attach protein molecules to the red cell by artificial means and to investigate the action of periodate on the attached protein by means of an antibody specific for the protein. The method used was based on the anti-globulin test described by Coombs, Mourant and Race (1945) for the detection of "non-agglutinating" *Rhesus* antibodies. Homozygous Rh-positive cells (DD) of group O sensitized with "non-agglutinating" anti-D serum were exposed to the action of 0·005 M periodate at pH 5 and 0·001 M periodate at pH 7 for various time intervals and were then tested for agglutination with anti-human-γ-globulin serum. Treatment with the more dilute periodate at pH 7 for 2 hours did not result in any lowering in the agglutination titre of the sensitized cells, but with 0·005 M periodate at pH 5·0 a seven-tube decrease in the end-point was observed after exposure of the cells to the reagent for only 15 minutes. Sensitized cells allowed to stand for a corresponding length of time in buffer solution at pH 5, in the absence of periodate, and subsequently washed twice with saline in the normal way, were agglutinated to the same extent by the anti-γ-globulin serum as were sensitized cells which had not been subjected to this treatment. The failure of the sensitized cells after treatment with periodate to react with the serum was therefore not due to the action of the acid buffer.

Precipitation reactions with anti-human-γ-globulin serum and solutions of purified human-γ-globulin (Kekwick and Mackay, 1949) (0·1 per cent), which had been allowed to react with 0·005 M periodate at pH 5 for various time intervals up to a total period of two hours, failed to reveal any loss of serological specificity.

DISCUSSION.

A study has been made of the action of sodium periodate under different conditions of pH and concentration on certain of the blood group receptors of

the human erythrocyte. The degree of inactivation has been measured by the fall in the capacity of the treated cells to react in simple agglutination tests with the appropriate serum and, in certain instances, by the power of the treated cells to absorb the specific antibody from the test serum.

The results obtained reveal that treatment of erythrocytes with periodate ultimately brings about the loss of all the specific serological characters examined. It is possible, however, by varying the concentration of periodate, time of reaction and pH, to demonstrate a differential inactivation of the group factors studied. For example, if the results obtained after treating the cells for 15 minutes with 0·001 M periodate at pH 5 are considered, it is evident that the specific group receptors can be divided into two categories—those susceptible to oxidation under these conditions and those resistant. To the former group belong the specific receptors M, N and *Rhesus* (D), and to the latter the structures specific for the gene products A, B, H, P, Le^a and O. A further differentiation of this latter group of receptors is brought about if 0·005 M periodate is used at pH 4 for 15 minutes. Under these conditions the A, B, H and O receptors are almost completely destroyed, whereas the P and Le^a structures are still detectable serologically. It is doubtful, however, whether the greater resistance of the P and Le^a receptors is sufficient to render this additional differentiation of practical importance. The results obtained at pH 7 confirm the observation of Moskowitz and Treffers (1950) that the A and B receptors are not appreciably inactivated by contact with 0·001 M periodate for 30 minutes, whereas the *Rhesus* (D) activity of the cells is largely destroyed.

The differences observed in the susceptibilities of the specific blood group structures to the action of periodate provide evidence that the failure of the cells to agglutinate with the specific blood group antibodies after exposure to the oxidizing agent is, in fact, due to inactivation or destruction of the specific receptors on the erythrocyte surface, and not due simply to other changes in the agglutinating properties of the cells ; if the latter were the explanation the rapidity with which loss of serological activity was observed would be expected to be constant irrespective of the blood group system under investigation. Further evidence that some abnormal state of the cells is not responsible for the lack of agglutination is provided by the fact that after treatment with periodate the cells are readily agglutinated by normal human sera (Stewart, 1949 ; Moskowitz and Treffers, 1950).

It is to be anticipated that the rate at which a susceptible blood group receptor is inactivated by periodate will be influenced not only by its chemical nature and structure, but also by the relative quantity and accessibility on the erythrocyte surface of the structures concerned. Nothing is known of the quantities of the various specific factors present, or of the spatial relationships of these determinant groups to other structures which compose the erythrocyte surface, and compared with the present knowledge of the chemistry of the water-soluble blood group substances, which have been identified as polysaccharide-amino acid complexes, relatively little is known concerning the chemical nature of the blood group receptors on the erythrocyte surface. The work of Hallauer (1934) and of Stepanov, Kuzin, Markageva and Kosjakov (1940) indicates that the A and B specific blood group materials obtained from erythrocytes are largely of a carbohydrate nature, and it seems reasonable to assume therefore that the water-soluble group substances (see Morgan, 1947*b* ; Kabat, 1949 ; Bray and Stacey, 1949) isolated

from tissue fluids and secretions constitute the specifically reactive materials on the erythrocyte surface. In consideration of the fact that the specific receptors on the cell surface are not removed, or are removed only with difficulty by repeated washing with saline, it may be assumed that these water-soluble group specific mucoids are combined with components of the cell membrane.

An investigation of the action of periodate on the specific blood receptors on the erythrocyte surface was undertaken in the hope that some insight would be gained into the nature of those group factors which do not occur to any appreciable extent in a water-soluble form, and which have not yet been identified chemically. Periodic acid differs from the common oxidizing agents in that it reacts mainly with compounds having two hydroxyl groups or a hydroxyl and an amino group attached to adjacent carbon atoms. This selectivity of action has led to a widespread application of the reagent for the determination of the constitution of carbohydrates. The complete inactivation of a specific blood group receptor on the erythrocyte surface, however, cannot be accepted as proof of its carbohydrate nature, for in addition to the reaction with α-glycol- and α-amino-alcohol groupings, oxidation of a number of amino-acids which do not contain these structures has been shown to occur (Jackson, 1944), although, admittedly, the reactions with these compounds proceed at a markedly slower rate. The nature of these reactions has not been established, but their existence makes it impossible to exclude substances from being protein in nature simply because their activity is lost on treatment with periodate.

Desnuelle and Antonin (1946) measured the formaldehyde liberated on treatment of a number of proteins with periodate, and found that the only protein that gave rise to a measurable quantity of formaldehyde was one which contained hydroxylysine, i.e., gelatin. It was therefore concluded that the reagent produced only minor changes in the proteins. Oxidative changes in the amino-acids which do not contain α-amino-alcohol groupings, however, fail to bring about the liberation of formaldehyde, and therefore would not be detected by this method. The observation that the toxicity of somatic antigens of *Shigella paradysenteriae*, which was believed to be associated with the protein component of the lipocarbohydrate protein complex, was diminished on treatment with periodic acid (Goebel, 1947), prompted this author to examine the action of the oxidizing agent on a number of biologically active compounds known to be protein in nature. The activity of crystalline ribonuclease against its specific substrate and of Type III pneumococcus immune globulin as measured by its power to precipitate with Type III polysaccharide were destroyed by treatment with the reagent, as was also the pathogenic action of the virus of Western equine encephalomyelitis (Goebel, Olitsky and Saenz, 1948). The loss of activity of the pneumococcus Type III immune globulin was also accompanied by changes in the absorption spectrum, but neither the species specificity of the protein nor the power to induce the formation of specific immune bodies was lost. In contrast the antigenicity of the equine virus was rapidly destroyed. A study of the physical, chemical and immunological changes in bovine serum albumin brought about by oxidation with lithium periodate (Goebel and Perlmann, 1949) revealed that destruction of certain amino-acids occurred, that the electrophoretic pattern was different after oxidation, that a change in the absorption spectrum was produced, and that prolonged treatment with lithium periodate destroyed the ability of the protein to induce the formation of antibodies. The treated albumin, however, was still

capable of precipitating some of the antibodies in sera of rabbits immunized with native serum albumin. It is therefore clear that, although the mechanism of the reactions involved has yet to be elucidated, significant changes in protein molecules can occur as a result of treatment with periodate ions. Nevertheless, for purposes of comparison with the results obtained for the blood group factors of unknown chemical composition, such as M, N, P and *Rhesus*, it appeared of interest to examine the action of the reagent on a surface component of the red cell known to be protein in nature. Attempts to do this, however, did not meet with success. Immunization of rabbits with a protein, stromatin, prepared from human erythrocyte stroma failed to elicit the formation of agglutinins for red cells, in spite of the fact that precipitins for stromatin were present in the serum. One might conclude from these observations that stromatin is not a serologically active component of the erythrocyte surface. The results obtained on treatment of *Rhesus* (D) cells sensitized with " non-agglutinating " antibody were inconclusive. The complete and rapid loss of activity of the sensitized cells against anti-γ-globulin serum after treatment with 0·005 M periodate at pH 5 is in sharp contrast to the results obtained with 0·001 M periodate at pH 7·0, where no inactivation was observed after 2 hours' treatment, and suggests changes occurring under the influence of the reagent other than oxidation of the attached γ-globulin. If the sensitized cells had not lost their capacity to agglutinate with anti-γ-globulin serum it would have been possible to state that the serological activity of the attached protein was unaffected by the oxidizing agent, but loss of activity cannot be accepted as proof that oxidative changes have occurred, in view of that fact that the bonds involved in antigen-antibody combination are labile and readily dissociated. Treatment of solutions of stromatin and γ-globulin with periodate under conditions of pH, time and concentration identical with those used for studying the action of the reagent on the red cell receptors did not result in loss of serological specificity as measured by their power to precipitate with the homologous antisera, but it need not be stressed that the rate of inactivation of a protein in solution is not necessarily comparable with the rate of inactivation of the same substance when it is present as a specifically orientated component of the red cell surface.

The results of the investigation do not allow a conclusion to be drawn as to the nature of those blood group factors which have not been identified chemically. It may be said, however, that those receptors whose specificity is almost certainly due to carbohydrate, A, B, H and Le^a, are inactivated more slowly by periodate than are the M, N and *Rhesus* D factors. Although a direct comparison between the rates of inactivation of the serological properties of carbohydrate and protein components of the erythrocyte surface has not been possible, it appears from the results of other experiments that proteins are on the whole less readily attacked than are carbohydrates. This being so, it will not be surprising if those receptors highly susceptible to treatment with periodate are subsequently found to be carbohydrate in nature.

SUMMARY.

(1) A study has been made of the action of periodate ions on a number of different specific blood group receptors on the human erythrocyte surface.

(2) The relative susceptibility of the receptors to the action of periodate was

measured by the loss of agglutinability with the homologous serum, and by the fall in the power of the treated erythrocytes to absorb the corresponding antibody from solution.

(3) The significance of the changes observed in the serological reactivity of the erythrocyte surface after exposure to periodate is discussed.

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A FAMILY SHOWING THE EXISTENCE OF THE RH GENE COMPLEX CD^{u}E.

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ATTENTION has been drawn to the allele D^u at the Dd locus by Stratton (1946). We have recently investigated a family in which the Rh gene complex CD^{u}E was found. This gene complex is an intermediate between Rz(CDE) and Ry(CdE), and must be extremely rare, as the estimated chromosome frequency percentage of Rz(CDE) is 0·24, and of Ry(CdE) is 0·0, calculated by the maximum likelihood method of Fisher from tests on 2,000 random bloods (Mollison, Mourant and Race, 1948).

At the time of testing the family consisted of mother (Mrs. R—), father and four children. The mother and all four children gave positive reactions with anti-C, anti-E and anti-c, and with some anti-D sera. The irregular reactions obtained with the anti-D sera (Table I) indicated the presence of the agglutinogen in the

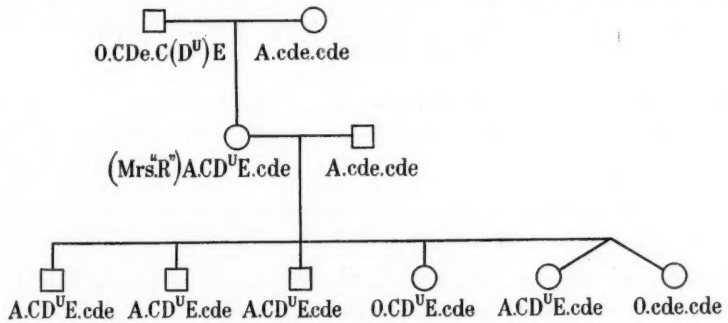

FIG. 1.

D^u form (Race, Sanger and Lawler, 1948). Since the father typed as Rh negative (cde/cde) the children must have inherited the C, D^u and E agglutinogens in the same gene complex from the mother. Therefore both they and the mother must be of the genotype CD^{u}E/cde.

In April, 1950, Mrs. R— was delivered of twins. One (Table I) was of genotype CD^{u}E/cde and the other was Rh negative (cde/cde). This last child was the only one in the family to inherit, and so confirm the existence of, the maternal cde gene complex.

TABLE I.—*The Rh Typing of the Red Cells of the R— Family.*

Red cells tested.	Testing Sera. Anti-D. 178.	218.	228.	241.	244.	267.	283.	286.	277.	284.	289.	287.	179.
Mr. R.—	–	–	–	–	–	–	–	–	–	–	–	–	–
Mrs. R—	–	++	++	–	–	++	+++	–	++	+	–	+	++
D. R—	–	++	+	–	–	++	+++	–	+	+	–	+	+
K. R—	–	++	+	–	–	+	+++	–	+	++	–	+	–
C. R—	–	++	+	–	–	+	++	–	+	+	–	++	++
V. R—	–	++	+	–	–	+	+++	–	+	+	–	++	++
Twin 1*	..	+	..	–	–	..	+++	..	..	..	..	++	++
Twin 2*	..	–	..	–	–	..	–	..	..	..	..	–	–
Positive control	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Negative control	–	–	–	–	–	–	–	–	–	–	–	–	–
Ditto	..	..	..	..	..	..	..	..	..	..	..	..	..

Red cells tested.	Testing Sera. Anti-C. 170.	297.	Anti-E. K19.	K21.	Anti-c. r_2.	r_8.	Genotypes.
Mr. R—	–	–	–	–	+++	+++	cdecde.
Mrs. R—	++	++	++	+++	++	+++	CD^{u}Ecde
D. R—	+	+++	+++	+++	+++	++	,,
K. R—	+	++	++	++	+	++	,,
C. R—	+	++	++	++	++	++	,,
V. R—	+	++	++	++	++	++	,,
Twin 1*	++	++	+++	+++	+++	++	,,
Twin 2*	–	–	–	–	+++	+++	cdecde
Positive control	++	+++	+++	+++	+++	+++	CDecDE
Negative control	–	–	–	–	..	..	cdecde
,, ,,	..	..	..	..	–	–	CDeCDe

* Born 4 months after the testing of the other children.

The parents of Mrs. R— were also tested. Her mother was Rh negative, but the father was of the phenotype C+D+E+c—. This means that he must have two C genes and so must have one gene complex containing both a C and an E gene. His reactions with anti-D serum were entirely normal, showing that he has at least one normal D gene. This means that the D^u gene which is probably present at the Dd locus is masked. It is almost certain, therefore, that he possesses a CD^{u}E gene complex (Fig. 1), and is probably of the genotype CD^{u}E CDe. There is a remote possibility, however, that the CD^{u}E gene inherited by Mrs. R— was the result of a mutation from either CDE or CdE, when his genotype would be either CDE CDe or CdE CDe.

We should like to thank our technician, Mrs. P. D. Pooley, for her interest and help in testing this family.

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